THE EFFECTS OF ELEVATED CARBON DIOXIDE ON GREENHOUSE GAS EMISSIONS FROM A TEMPERATE PASTURE

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There is nothing like looking,

if you want to find something.

You certainly usually find something if you look,

but it is not always quite the something you were after.

--J.R.R. Tolkien

ABSTRACT

Global surface temperatures have been rising since the pre-industrial era and are predicted to continue rising due to the positive radiative forcing of greenhouse gases in the atmosphere. The atmospheric concentration of carbon dioxide (CO_2), the gas responsible for most of this warming, has increased since the industrial revolution due to the burning of fossil fuels and land use change. The concentration of nitrous oxide (N_2O), a potent greenhouse gas with substantially higher global warming potential than CO_2 over a 100-y horizon, has similarly increased due to agriculture and nitrogenous fertiliser use. In addition to warming the earth's surface, elevated CO_2 (eCO_2) may change terrestrial carbon (C) and nitrogen (N) cycling due to plant responses to increased C availability. Plants respond to eCO_2 in two ways: through increased biomass production and increased water-use efficiency. These responses may alter the availability of C, N and water in soils, which could affect microbial activity through shifts in substrate availability and soil aerobic status. As many processes mediated by microbes release CO_2 and N_2O, it is likely that both plant responses to eCO_2 could alter these gas fluxes. Soils are the largest source of CO_2 and N_2O, making it essential that accurate predictions of future greenhouse gas emissions include the response of these processes to eCO_2.

Using a temperate pasture dominated by *Lolium perenne*, this study measured the impact of three levels of CO_2 concentration (400, 475 and 550 µmol CO_2 mol^{-1}) on the emission of CO_2 and N_2O. Additionally, water supply was independently controlled via irrigation treatments (adequate, excess = +20% and limit = -40%) to determine if CO_2-effects on soil processes were driven by plant production, soil water content or both. Plant biomass was increased under 550 µmol CO_2 mol^{-1} relative to ambient due entirely to greater root growth but no similar change in production was present under 475 µmol CO_2 mol^{-1}. Plant N requirements were increased under eCO_2 relative to ambient, resulting in additional N immobilised in plant

biomass under eCO_2 and substantially lower soil mineral N availability. Consequently, N_2O emissions were never higher under eCO_2 than ambient and were often lower. This effect dramatically reduced N_2O emissions from this pasture with the strongest effect in the week following fertiliser application when emissions were highest. Under eCO_2, soil CO_2 emissions were higher than at 400 µmol CO_2 mol^{-1}, though mean emissions were similar between 475 and 550 µmol CO_2 mol^{-1}. Similarly, the rate of root decomposition in soils under eCO_2 was higher than in control plots as was the loss of soil C. The CO_2-effect on both CO_2 and N_2O emissions was independent of soil water content, demonstrating that the CO_2-effect on plant production and organic matter inputs drove the processes underlying these fluxes.

Therefore, eCO_2 had a strong effect on the emission of CO_2 and N_2O from this temperate pasture. In particular, N_2O emissions were suppressed by the CO_2-effect on plant N immobilisation and CO_2 emissions were accelerated due to increased belowground C input. Importantly, the effects of soil water content on these emissions were absent in this system entirely, suggesting that the CO_2-effect on plant production can have a substantial impact on greenhouse gas emissions from temperate pastures.

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1 GENERAL INTRODUCTION

The transformation and exchange of elements through biogeochemical pathways form the basis upon which a finite supply of resources in the Earth system are recycled between terrestrial ecosystems and the atmosphere. Carbon (C) and nitrogen (N) are essential for life on Earth as C is the building block of organic matter and N is required for the synthesis of amino acids and proteins. The pools of C and N in the atmosphere and in soils are vast relative to biological demand but are inaccessible to many organisms without prior transformation mediated by plants or microbes. Consequently, the supply of nutrients in ecosystems is regulated by plant- and microbially-mediated C and N cycles (Schlesinger 1991, Sterner and Elser 2002). Plants assimilate atmospheric carbon dioxide (CO_2) into organic matter via photosynthesis but are unable to similarly fix the vast supply of atmospheric dinitrogen (N_2). Rather, plants mostly depend on the limited supply of mineral N made available through microbially-mediated decomposition of plant and animal organic matter, although some plants maintain a symbiotic relationship with nitrogen-fixing prokaryotes (Belnap 2003). Decoupling the C and N cycles is difficult as microbial activity is dependent on the supply of organic matter from plants just as plant production depends on mineral N made available by microbes. Therefore, changes to either the C or N cycle can impact productivity and ecosystem function. Rising atmospheric CO_2 concentration ($[CO_2]$) may influence these cycles by both changing the climate via positive radiative forcing (Stocker 2014) and by increasing the availability of C for plant production (Gifford 1992). However, mineral N typically limits production in terrestrial systems (Vitousek and Howarth 1991). Therefore, the extent to which $[CO_2]$ stimulates plant production may be determined by the response of N availability to $[CO_2]$ (Reich et al. 2006b). Additionally, shifts in soil C and N supply may alter microbial processes, several of which produce nitrous oxide (N_2O), a potent greenhouse gas. Therefore, the understanding of ecosystem productivity and future

climate conditions requires investigation of coupled C and N cycle responses to rising atmospheric [CO_2].

1.1 Carbon and nitrogen cycling

Plants assimilate C from atmospheric CO_2 into organic molecules through photosynthesis (Fig. 1.1 Process 1), the largest flux of C in Earth's biosphere (Schlesinger and Andrews 2000) and the primary source of ecosystem energy (Field et al. 1998). These organic compounds enter the soil through animal remains or excretion (Parham et al. 2002), litterfall, root exudation and root mortality (Walker et al. 2003, Shahzad et al. 2015a), providing soil microbes with organic C and N (Fig 1.1 Process 3). Simple organic molecules such as root exudates can turnover rapidly through microbial metabolism, while more recalcitrant forms of organic C are resistant to microbial breakdown and can remain stable in soil aggregates. Fixed C is then returned to the atmosphere through a variety of microbially-mediated pathways. In aerobic soils, heterotrophic microbes oxidise organic matter, using some of this C for their biomass while the rest is released as CO_2 via heterotrophic respiration (Fig. 1.1; Gougoulias et al. 2014). Root production and maintenance leads to a plant-derived respiratory CO_2 release from soil (Fig. 1.1 Process 2), termed soil autotrophic respiration (Fig. 1.1 Process 2; Kuzyakov and Larionova 2005). The sum of these two fluxes are called soil respiration (R_S), which is the second largest flux of C in the biosphere (Schlesinger and

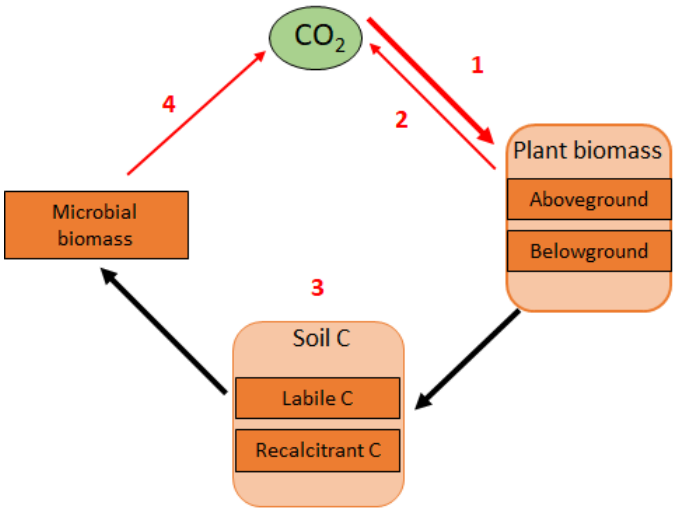

Figure 1.1. Simplified conceptual diagram of the terrestrial carbon cycle. Red arrows indicate processes that were measured in this study. Bold arrows indicate processes affected by eCO_2 based on the literature.

Andrews 2000). Microbes can also release methane (CH_4), a potent greenhouse gas, via decomposition in waterlogged soils (Lu and Conrad 2005). As most soils are oxic, CO_2 is the primary gas produced during the decomposition of organic C (Schlesinger and Andrews 2000).

Microbes also mediate the mineralisation of organic N into inorganic forms, which is the main source of N for plants (Weigelt et al. 2005, Haynes 2012). In aerobic soils, organic matter is transformed via ammonification into ammonium (NH_4^+; Fig. 1.2 Process 1), which can then be immobilised in biomass (Fig. 1.2 Process 2 and 3) or oxidised to nitrate (NO_3^-) via nitrification (Fig. 1.2 Process 5). Mineralisation rates are strongly influenced by the quality of the substrate due to microbial C and N requirements (Janssen 1996, Jin et al. 2013). High C:N organic matter results in assimilation of scavenged N into microbial biomass, while low C:N organic matter results in greater release of inorganic N as microbial needs are readily met (Janssen 1996, Robertson and Groffman 2007). Although mycorrhizal fungi can provide plants with N, availability of NH_4^+ and NO_3^- often limits production in terrestrial systems, and therefore the availability of mineral N via organic matter mineralisation is crucial for ecosystem function (Vitousek and Howarth 1991, Elser et al. 2007).

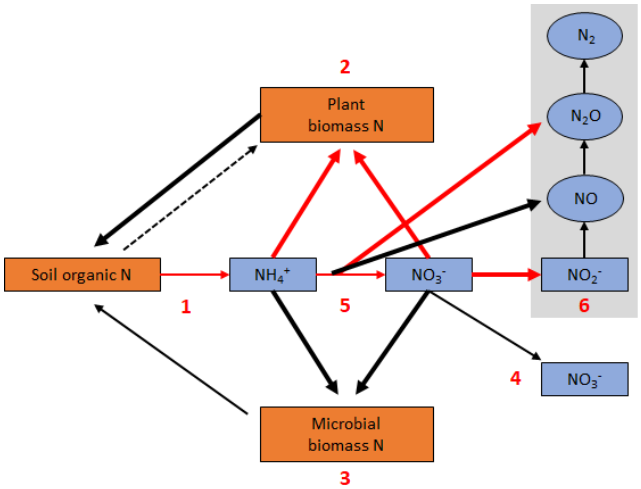

Figure. 1.2. Conceptual diagram of the terrestrial N cycle. Gases are in ovals. Red arrows indicate processes that were measured in this study. Bold arrows indicate processes affected by eCO_2 based on the literature.

Microbially-mediated pathways in the N cycle influence the loss of N from soil and produce N_2O, a potent greenhouse gas (Fig. 1.2). In many production systems, including managed pastures, nitrogenous fertiliser is applied to alleviate N constraints on primary production (Ball and Ryden 1984). However, high application rates in pastures can exceed plant N requirements, resulting in urea-N loss from 1.6% (Luo et al. 2007) to 15% (Ball and Ryden 1984). Fertiliser is often applied as urea or other organic forms which are rapidly mineralised in pastures, where ammonification and nitrification rates are typically high (Haynes 2012). NO_3^- is vulnerable to leaching (Fig. 1.2 Process 4), the transport of minerals through the soil profile in solution, as anions do not adsorb to the negatively charged soil particles (Dinnes et al. 2002). Leaching increases loss of mineral N from soil and leads to eutrophication of waterways (Di and Cameron 2002). Denitrification (Fig. 1.2 Process 6) is often a major N transformation pathway in agricultural soils due to the abundance of NO_3^- (Barton et al. 1999, Reay et al. 2012, Beeckman et al. 2018). Under anaerobic conditions with available organic matter, denitrifiers transform oxidised forms of N such as NO_3^- and NO_2^- into increasingly reduced intermediary forms i.e. nitric oxide (NO), nitrous oxide (N_2O) and finally dinitrogen (N_2; Knowles 1982, Firestone and Davidson 1989b). These gases can be released from the soil, taking with them soil N and contributing to climate change (van der Salm et al. 2007, Schlesinger 2009).

1.2 Global change

Global surface temperatures have risen by approximately 1.0°C above pre-industrial levels and are expected to continue rising due to the positive radiative forcing of greenhouse gases in the atmosphere (Stocker 2014).The atmospheric concentration of CO_2, the greenhouse gas responsible for most of the positive radiative forcing, has increased from 277 ppm in the pre-industrial era to 405 ppm in 2017 (Le Quéré et al. 2018). Anthropogenic CO_2 emissions before 1950 were primarily driven by land use changes (Ciais et al. 2013), but from 2008–

2017, 87% of anthropogenic CO_2 emissions were released from the burning of fossil fuels and industry (Le Quéré et al. 2018). The greenhouse gas CH_4 is the second largest contributor to positive radiative forcing after CO_2 and is produced in anaerobic environments such as rice paddies and livestock as well as through industrial practices (Saunois et al. 2016). The concentration of N_2O, the third most important greenhouse gas, has risen since pre-industrial levels from 270 ppb to 324 ppb (Ravishankara et al. 2009). The atmospheric [N_2O] is substantially lower than [CO_2], yet it contributes to 6% of climate change due to positive radiative forcing, long atmospheric lifespan of 118-131 years (Fleming et al. 2011), and ozone-depleting properties (Ravishankara et al. 2009). Overall, the greenhouse gas potential of N_2O is 298 times that of CO_2 over a 100 year period (Stocker 2014). Soils are the largest source of N_2O, with 4.1 Tg N_2O-N yr^{-1} emitted from agricultural land, due largely to nitrogenous fertiliser application, and 6.6 Tg N_2O-N yr^{-1} from soils with natural vegetation (Thomson et al. 2012). In both natural and managed systems, biological processes within the soil contribute strongly to overall fluxes of all these greenhouse gases.

The C and N cycles are sensitive to environmental perturbations, thus global change will likely alter the plant- and microbially-mediated processes regulating ecosystem exchange of these gases. One major driver of global change is elevated CO_2 (eCO_2), which may alter coupled C and N cycles through plant responses to increased C availability (Schimel 1995). Under eCO_2, plant photosynthesis is increased (Ainsworth and Rogers 2007), as is plant biomass and organic matter deposition via litterfall (de Graaff et al. 2006). Furthermore, plants allocate additional C belowground to root production (de Graaff et al. 2006, Iversen et al. 2008), thereby directly affecting soil CO_2 flux via root respiration (Luo et al. 1996) but also by increasing microbial access to substrate via root exudation and turnover (Pendall et al. 2004, Iversen et al. 2008) and higher rates of root sloughing (Madhu and Hatfield 2013). Despite increased fixation of atmospheric C into plant biomass, it remains unclear to what

extent C storage in soils will be changed (van Groenigen et al. 2014). The CO_2-effect on belowground labile C inputs can accelerate microbially-mediated decomposition of soil organic matter through microbial 'priming' (Cheng and Johnson 1998). Therefore, eCO_2 may stimulate the mineralisation of C from soils as well as its incorporation, limiting C accumulation in soils and making forecasts of soil C content problematic.

By altering plant tissue chemistry, eCO_2 may impact the rate at which organic N is mineralised, thereby altering N availability. eCO_2 increases plant tissue C:N (Cotrufo et al. 1998), which may similarly increase soil C:N (Stiling and Cornelissen 2007). Increased soil C:N may stimulate microbial immobilisation and lower mineralisation rates (Janssen 1996). Consequently, mineral N availability may be reduced under eCO_2 as the input of new inorganic N declines. eCO_2 may further exacerbate mineral N availability through greater immobilisation of N in biomass (Luo et al. 2004). These declines could increase N constraints on primary production as well as microbial processes dependant on mineral N. Heterotrophic denitrifiers require oxidised forms of N (e.g. NO_3^-) to be used as an electron acceptor during the oxidation of an electron donor (e.g. organic matter; Robertson and Groffman 2007). Consequently, eCO_2 may accelerate denitrification through increased belowground input of organic matter to heterotrophs (Baggs et al. 2003a, van Groenigen et al. 2011). Alternatively, increased immobilisation of mineral N in plant and microbial biomass or recalcitrant soil organic matter pools could constrain denitrifier access to NO_3^- (Hungate et al. 1997c, Kettunen et al. 2007, Sun et al. 2017). Disentangling the effects of eCO_2 on soil C and N availability will help to predict future N_2O emissions.

eCO_2 may further impact N cycling through indirect shifts in soil water content. Rising atmospheric [CO_2] is expected to increase soil water content in vegetated soils due to reduced stomatal conductance and transpiration (Leakey et al. 2009). Microbially-mediated processes are influenced by soil oxygen content, and therefore may be sensitive to these changes in soil

water content. Mineralisation of organic matter requires oxidative soil conditions and consequently is reduced in anaerobic soils (Agehara and Warncke 2005, Robertson and Groffman 2007). Conversely, lower soil oxygen will increase the favourability of NO_3^- as an electron acceptor, potentially stimulating denitrification mediated by heterotrophs (Bateman and Baggs 2005). Such a shift could result in lower availability of inorganic N by suppressing mineralisation of organic matter but also by stimulating dissimilatory reduction of NO_3^- to NO, N_2O and N_2. Consequently, the indirect effect of eCO_2 on soil water content may lower N availability for production and increase emission of N_2O.

Climate change will undoubtedly affect soil water content further through shifts in precipitation patterns. Models predict positive radiative forcing due to greenhouse gases will increase drought prevalence in mid- and low-latitude regions (Seager et al. 2007, Dai 2011, Trenberth et al. 2013) and reduce the frequency of rainfall (Allan and Soden 2008, Singh et al. 2013). Consequently, soils will likely be drier and go for extended periods of time without rainfall. Low soil water content can affect microbial processes by changing soil aerobic status, induce osmotic stress (Schimel et al. 2007) and restrict access to substrate (Larsen et al. 2011, Sheik et al. 2011). However, shifts in plant-water relations with [CO_2] may help to mitigate the effects of soil drying through greater soil water retention (Leakey et al. 2009). As both changing precipitation patterns and plant responses to [CO_2] may impact water availability and therefore affect microbial processes, investigation of these factors in combination is essential for accurate predictions of future C and N cycling

1.3 Research aims

The objective of this study was to investigate the impact of rising atmospheric [CO_2] on biogeochemical processes leading to CO_2 and N_2O emissions from an intensively managed temperate pasture. The results of previous pasture studies have been mixed, with some

reporting a suppression of N_2O emissions under eCO_2 (Hungate et al. 1997c, Kettunen et al. 2007) while others found an acceleration (Baggs et al. 2003a, Kammann et al. 2008, Zhong et al. 2015). Similarly, C storage in grasslands under eCO_2 has been shown to increase in some cases (de Graaff et al. 2006, Luo et al. 2006) while being reduced in others (van Groenigen et al. 2014). There are an estimated 28 million km^2 of pasture worldwide, an area occupying 22% of the Earth's ice-free land surface (Ramankutty et al. 2008). Consequently, the CO_2 and N_2O emissions from managed pastures comprise a substantial proportion of the global greenhouse gas budget. Additionally, in Australia, meat and wool production from grazing animals account for 20% of the gross value of agricultural products in Australia (Moore and Ghahramani 2013). Thus, CO_2-mediated shifts in future pasture production could impact this industry and food availability.

1.4 Thesis structure

- In Chapter 2, I describe the design and implementation of TasFACE2, the experimental site where this study was performed.
- In Chapter 3, I determine to what extent mineral N availability is altered by [CO_2] and water supply and assess whether those shifts are due to changes in mineralisation, immobilisation or other losses.
- In Chapter 4, I determine the response of N_2O emissions to [CO_2] due to plant-mediated shifts in C, N and water availability.
- In Chapter 5, I determine the response of CO_2 emissions and root decomposition to [CO_2] across a range of soil water content.
- In Chapter 6, I synthesise the findings from the previous chapters, discussing the integrated effects of [CO_2] and water supply on soil C storage, N availability as well as emissions of CO_2 and N_2O from this pasture.

2 METHODS

2.1 Study site and experimental design

2.1.1 Site details and site preparation

I performed this study in the TasFACE2 experiment (Brinkhoff et al. 2019), which is situated on a long-term pasture used for sheep grazing, dominated by *Dactylis glomerata, Lolium perenne* and *Trifolium subterraneum* in Cambridge, Tasmania, Australia (42°48'S, 147°25'E; 50 m a.s.l.), where the annual mean maximum temperature is 12.1°C and a mean annual rainfall of 598 mm (Australian Bureau of Meteorology 2018). The soil at the site is strongly duplex, consisting of grey-brown sandy loam to 30 cm on sandy medium to heavy clay with a pH ~6.5.

During the autumn of 2015, a 50 × 40 m site was thoroughly irrigated for a period of approximately one month to encourage vigorous growth and then was sprayed with a post-emergence herbicide to kill all existing plants. Once the existing herbage had died completely, all aboveground biomass was removed by mowing. The entire site was then sown by direct drilling with a monoculture of *Lolium perenne* cv. Base AR37 (PGG Wrightson Seeds, Truganina, Vic. Australia) at a sowing rate of 50 kg ha^{-1}, which is roughly double the recommended sowing rate. Nutriphos fertiliser (N:P:K 16:20:0, Hellagrolip, Athens, Greece) was applied at sowing to provide 20 kg N ha^{-1}.

During May and June (winter) 2015, twelve 3 × 3 m square experimental plots were established in a random block design with four blocks of three plots, with a buffer zone of 7.5 m between each plot and block. In the centre of each 3 × 3 m square, a 1.8-m diameter pentagonal experimental plot was established by trenching (10 mm width) to ~900 mm depth. Each plot was divided into five equal radial segments by trenching to ~900 mm to give five

209 0.5 m^2 sectors in each plot (Fig. 2.1). Each sector was wrapped with PVC film to a depth of
210 ~700 mm prior to backfilling all trenches with site soil, carefully preserving soil horizons. As
211 the rooting depth at this site is ~400 mm, this method effectively isolated each sector from
212 adjacent sectors and from the surrounding soil, allowing local soil moisture conditions to be
213 controlled.

Figure 2.1. Photo of one plot area (left) and a shelter shortly after establishment of the experimental plots and CO_2 fumigation commenced in early spring 2015.

214 *2.1.2 Soil water supply*

215 Rainfall was excluded from each plot by installing a sloping, UV-transparent polycarbonate
216 rainout shelter at a height of 1.7 m at the high side and 1.4 m at the low side (Fig. 2.1). Plots
217 were watered twice weekly by an automated irrigation system with five fixed-flow drippers
218 evenly spaced across each sector to achieve relatively uniform soil moisture levels within
219 each sector. Irrigation amounts were determined by modelling evapotranspiration rates using
220 the EcoMod pasture simulation (Perring et al. 2010) and long-term weather data for the site,
221 parameterised for *L. perenne* exactly as in Perring et al. (2010). Evapotranspiration was
222 estimated daily but averaged over the month to obtain monthly irrigation volume. Irrigation
223 treatments delivered to sectors were *sufficient* water (aimed at supplying the total
224 transpirational demand), *limit* (40% less water supplied than in sufficient sectors) or *excess*
225 (20% more water supplied than sufficient plots). Three of the five sectors were designated a

seasonal watering treatment, which varied the volume of water supplied in spring, summer and autumn (e.g. *limit* spring, *sufficient* summer, *excess* autumn) and supplied *sufficient* during the winter period (1 June – 31 August; Table 2.1). The other two sectors received *sufficient* water in all months. One of those sectors was amended with a nitrification inhibitor separate from this thesis. Only results from the sectors supplied with seasonal irrigation treatments and the *sufficient*-only without the nitrification inhibitor are included in this thesis. The results of TasFACE1 demonstrated winter precipitation had no impact on the CO_2-effect on productivity, whereas precipitation volume in other seasons had a strong influence (Hovenden 2014). TasFACE2 builds on those results by specifically investigating the influence of precipitation in spring, summer and autumn on production. Uniform watering over winter is essential for determination of these influences. Further, seasonal irrigation treatments had little impact on the variables measured in this thesis. As such, treatments were grouped by month according to the irrigation treatment at the time. Soil moisture was monitored continuously throughout the experiment using CS616 water content reflectometer probes (Campbell Scientific, Port Macquarie, Australia) buried horizontally at 20 cm depth in each sector and logged by a CR1000 data system (Campbell Scientific, Port Macquarie, Australia). Surface soil moisture content was also collected manually, twice weekly using a FieldScout 300 time-domain reflectometry probe (Spectrum Technologies, Aurora, IL, USA).

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Table 2.1. TasFACE2 irrigation treatment water supplied to sectors. Each sector received *sufficient* water (aimed to supply transpirational demand), *limit* (-40%) or *excess* (+20%). Values indicate the water supplied (mm) per watering event, of which there were two per week. Quantities were adjusted monthly. All treatments were adjusted to *sufficient* in winter (June-August).

Season	Month	*Sufficient*	*Excess*	*Limit*
Summer	December	12.6	15.8	7.6
	January	11.6	14.5	6.9
	February	9.0	11.3	5.4
Autumn	March	7.6	9.5	4.6
	April	6.5	8.1	3.9
	May	4.7	5.9	2.8
Winter	June	3.6	3.6	3.6
	July	5.1	5.1	5.1
	August	6.9	6.9	6.9
Spring	September	7.2	9.0	4.3
	October	13.4	16.7	8.0
	November	11.3	14.1	6.8

2.1.3 Free-air carbon dioxide enrichment (FACE)

Each of the twelve experimental plots was allocated to one of three [CO_2] - 400 μmol mol^{-1} (control), 475 μmol mol^{-1} or 550 μmol mol^{-1}- such that each block contained one plot of each [CO_2] (Fig. 2.2). Fumigation treatments commenced in June 2015, with [CO_2] being controlled in the 475 and 550 μmol CO_2 mol^{-1} plots using pure-CO_2 free-air carbon dioxide enrichment (FACE) technology, exactly as in Hovenden et al. (2006). Briefly, pure CO_2 was released via a circular, 1.8 m diameter PVC ring containing 0.3 mm laser-drilled holes every 5 cm along its length (Fig. 2.3). Each such ring was suspended above the periphery of the pentagonal experimental area in each plot at a height ~10 cm above the sward with the ring height adjusted weekly to account for pasture growth and harvesting. The concentration of CO_2 in the centre of each experimental plot was monitored continuously by infrared gas analysis (SMA-4, PP Systems, Amesbury, USA) and the rate of CO_2 release was controlled via proportional valves. The flow through each valve was controlled by manipulating the voltage (0 – 10 V DC) supplied to the valve using a modified Proportional Integration Device algorithm integrating [CO_2] and wind velocity. Fumigation commenced at sunrise and ceased at sunset each day. Valves were not closed at high wind velocities, as occurs in some other FACE experiments. All variables (ring [CO_2], valve voltage, windspeed) were logged each second using a personal computer.

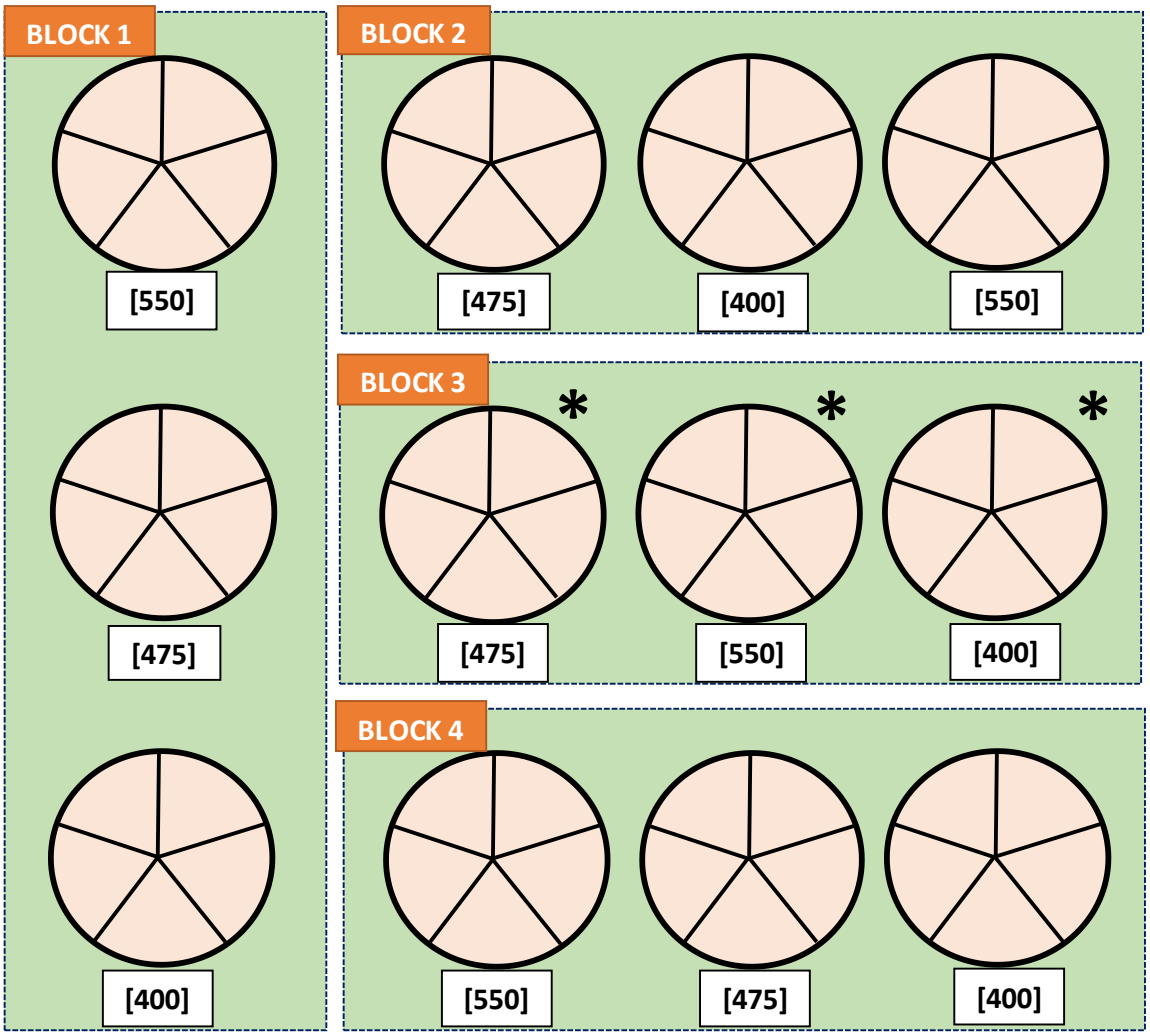

Figure 2.2. Simplified schematic of the TasFACE2 random block design experiment. Bracketed numbers indicate plot CO_2 concentration (approximately 400, 475 and 550 µmol CO_2 mol^{-1}). Asterisks (*) indicate every sector of the plot contained a permanent soil moisture probe buried horizontally at 20 cm. Each radial segment was approximately 0.5 m^2 and received one of three irrigation treatments (*sufficient*, *limit*, *excess*).

Figure 2.3. Photo of one plot area (left) and the paddock with several shelters (right) in autumn 2016.

2.2 Site measurements

2.2.1 Biomass and plant nutrients

Aboveground biomass was harvested by clipping once substantial pasture growth had occurred in the spring of 2015. Aboveground biomass was then harvested regularly, when plants had reached the three-leaf stage, which occurred approximately monthly year-round. When harvested, the entire area of each plot was clipped to a height of approximately 4 cm and clipped material was retained for nutrient analysis and biomass estimation. Immediately following each harvest, a single application of granular urea (Impact Fertilisers, Melbourne, Australia; 46-0-0-0: N-P-K-S) was applied manually by hand at a rate of 2.3 kg urea-N ha^{-1} d^{-1} since previous application. Superphosphate was applied every six months at a rate of 25 g m^{-2}. *L. perenne* biomass yields increase nearly proportionally with N application up to approximately 1.5 kg N ha^{-1} d^{-1}, beyond which biomass yields and plant N uptake are relatively unchanged (Rawnsley et al. 2014). All urea was expected to be converted to NH_4^+ within 2-5 days of application.

Root production was estimated using root ingrowth cores (Nadelhoffer and Raich 1992). For each sector, three root ingrowth cores (height 10 cm, diameter 3 cm) were filled with sieved, root-free bulk site soil (0 – 10 cm) before being inserted into gaps created using a 3 cm soil augur. Ingrowth cores were extracted and replaced at approximately 60 d intervals. In preparation for elemental analysis, I soaked roots in a 5% sodium hexametaphosphate solution (aggregate dispersal agent) for approximately 24 h before rinsing with deionized water. Root material from these cores was bulked at the sector level for analysis. Leaf material from harvests and root material from ingrowth cores was dried at 50°C for 24 h before being weighed. Dried plant material was ground to powder in a ball mill and analysed for C and N in an elemental analyser (Perkin Elmer, Melbourne, VIC, Australia).

Importantly, this study is part of a larger project investigating biomass production at the same experimental site, TasFACE2. This thesis is focused on greenhouse gas emissions from TasFACE2 rather than the biomass response to treatment. As such, biomass data are referenced for interpretation of the findings presented in this study but are not presented in detail in this thesis. Production data are available in the Supplementary Material (Fig. S1) but are not presented in full.

3 EFFECTS OF CARBON DIOXIDE CONCENTRATION ON SOIL MINERAL NITROGEN AVAILABILITY

3.1 Introduction

Nitrogen (N) is one of the most important elements for ecosystem functioning as it is an essential component in amino acids and proteins (Canfield et al. 2010). It is also the most abundant element in our atmosphere, with dinitrogen (N_2) comprising most of this pool (Robertson and Groffman 2007). Plants are unable to directly access atmospheric N and instead depend on inorganic N, typically occurring in the soil as nitrate (NO_3^-) and ammonium (NH_4^+;Elser et al. 2007), low molecular weight organic molecules (Jones et al. 2005, Paungfoo-Lonhienne et al. 2008) or microbially-fixed N (Robertson and Groffman 2007). Due to the high demand for N by plants and microbes, N availability commonly limits ecosystem productivity (Gruber and Galloway 2008), hence the importance of nitrogenous fertilisers for maximising agricultural production (Mosier et al. 2013). Given the importance of N availability for ecosystem function, it is essential to understand the processes impacting the size of the various N pools and the rates of flux among these pools, particularly in light of changing environmental conditions (Reich et al. 2006b).

A variety of biogeochemical pathways regulate the flux of N between organic and inorganic pools in the N cycle (Fig. 1.2). In a process called ammonification, organic N contained in biomass and soil organic matter is metabolised as an energy source by soil-dwelling bacteria and fungi which release NH_4^+ (Robertson and Groffman 2007). Nitrification is an oxidative process performed in two steps in which NH_4^+ is transformed to nitrite (NO_2^-) and finally NO_3^-, predominantly by ammonia oxidizing bacteria and archaea followed by nitrite oxidising bacteria, or in a single step by commamox bacteria (Beeckman et al. 2018). Some fungi are also capable of nitrification through the oxidation of NH_4^+ and organic N to NO_3^-

(Schimel et al. 1984, Laughlin et al. 2008, Maeda et al. 2015). The series of transformations which oxidise organic N into inorganic N are termed mineralisation and occur primarily in aerobic environments (Robertson and Groffman 2007). Mineral N can be immobilised in plant and microbial biomass or it can be lost from the soil through gaseous emissions or leaching (Bateman and Baggs 2005). In anaerobic conditions, denitrifying microbes reduce oxidised mineral N such as NO_3^- and NO_2^- into increasingly reduced forms such as nitric oxide (NO), nitrous oxide (N_2O) and N_2 which can be lost from soil as gas (Davidson et al. 2000). Soil N may also be lost via leaching when mineral N is mobilized in saturated soils (Di and Cameron 2002). Cations such as NH_4^+ bind to negatively charged soil particles, while anions such as NO_3^- are readily transported in soil solution to groundwater (Di and Cameron 2002). The availability of mineral N in soils is determined by the inputs via mineralisation balanced against soil losses due to biological immobilisation, leaching and gaseous emissions.

The physical and biogeochemical processes governing N pool sizes are influenced by environmental conditions and therefore may be altered by global change. Elevated CO_2 (eCO_2) may impact N cycling via two plant responses which likely will alter ecosystem C, N and water availability (Reich et al. 2006b). First, stomatal conductance is reduced under eCO_2, which lowers plant water use and consequently increases soil water content (Leakey et al. 2009). High soil moisture content can potentially both reduce inputs via mineralisation and increase losses via denitrification and leaching. Mineralisation rates are highest between 30-65% water-filled pore space (WFPS) but decrease rapidly above or below this range (Cassman and Munns 1980). Heterotrophs will use oxidised forms of N such as NO_3^- only when oxygen is unavailable. Thus, denitrification and associated gas losses increase as WFPS increases (Bateman and Baggs 2005). Losses of N via leaching increases with soil water content due to increased mobilisation of ions in solution (Di and Cameron 2002). Second,

eCO_2 suppresses photorespiration and increases the rate of RuBP carboxylation, therefore increasing plant C assimilation rate (Leakey et al. 2009).This additional photosynthate supply increases the provision of C-rich root exudates (Bertin et al. 2003, Shahzad et al. 2015a) and sloughed roots (Iversen et al. 2008) to microbes, which could stimulate the organic matter mineralisation rate (Phillips et al. 2012, Zhu et al. 2014, Hasegawa et al. 2016). eCO_2 can lead to higher C:N of plant tissues in some cases, which can alter the C:N of soil organic matter (Norby et al. 2001, Silver and Miya 2001, Stiling and Cornelissen 2007). Soil organic matter C:N strongly affects ammonifying and nitrifying microbe activity as they depend on N-rich organic matter to meet physiological requirements (Janssen 1996). However, the changes in C:N associated with eCO_2 may be minor relative to other factors such as soil water content and may have little impact on mineralisation (Jin et al. 2013, Osanai et al. 2015). Importantly, greater production of plant biomass with high C:N under eCO_2 can lead to the immobilisation of N in plant tissues, microbial biomass and recalcitrant soil organic matter, effectively lowering available mineral N (Luo et al. 2004, Reich et al. 2006a).

Plants have a strong influence on ecosystem C and N availability through organic matter production and alter soil water content through water use. Therefore, the $[CO_2]$ response curve for the processes regulating plant use of C, N and water will likely affect ecosystem availability of these resources. The C_3 plant water-use efficiency response curve is positive, linear and is nearly proportional to $[CO_2]$ (Polley et al. 1993, Anderson et al. 2001a), suggesting soil water content may similarly increase with $[CO_2]$. C and N availability may also be altered due to positive, linear responses to $[CO_2]$ such as photosynthesis, tissue C:N and soil C:N (Gill et al. 2002a), potentially impacting microbially-mediated processes. In fact, Gill et al. (2002a) demonstrated N mineralisation rates decline with $[CO_2]$ but a non-linear trend indicates the change is less severe at $[CO_2] > 400$ μmol CO_2 mol^{-1}. Thus, it is unclear to what extent modest changes in mineralisation above 400 μmol CO_2 mol^{-1} will alter

N availability in a managed grassland. Furthermore, it is difficult to distinguish the role of plant responses to [CO_2] on N availability. Given the CO_2-effect on plant production may directly lower N availability through increased plant and microbial N immobilisation (Luo et al. 2004) or the indirect CO_2-effect may lower soil aerobic status, thereby reducing mineralisation rates and stimulating denitrification (Baggs et al. 2003a). Thus, disentangling the indirect impacts of plant water-use efficiency and the direct impacts of plant production on N availability requires independent control of water availability and [CO_2]. If the CO_2-effects persist across a range of soil water contents, then those responses can be attributed to CO_2-induced changes in production and organic matter input. If soil water content is shown to influence soil N availability, then either water availability or a combination of water availability and plant production drive the CO_2-effect on soil N availability.

Here I analyse the independent and combined effects of [CO_2] and soil water supply on mineral N availability in a temperate grassland using the TasFACE2 experiment. I used three levels of [CO_2] (400, 475 and 550 μmol CO_2 mol^{-1}) and three levels of water supply (*sufficient*, *limit*, and *excess*) to assess changes in N cycling. I hypothesised that CO_2-induced changes in plant production and tissue chemistry rather than soil water content would drive mineral N availability in this grassland. I tested this by measuring the change in NH_4^+ and NO_3^- availability in response to independently controlled [CO_2] and soil water supply. If the CO_2-effect persists regardless of soil water content, then these responses are driven by the CO_2-effect on C cycling rather than plant water use. I also set out to determine if mineral N availability responded linearly to [CO_2] and assessed whether any changes were due to a CO_2-effect on mineralisation, immobilisation or lost through another pathway.

3.2 Methods

3.2.1 Soil nutrients

Available NH_4^+ and NO_3^- was measured using anion and cation ion-exchange membranes (IEM; Abrams and Jarrell 1992, Bowatte et al. 2007, Hovenden et al. 2008). IEM (VWR International Pty Ltd, Murarrie, Australia) were 10 mm × 50 mm and were either positively or negatively charged. One of each charge was affixed to a plastic tag for installation in soil. Prior to installation in soil, IEM were soaked in 2 *M* KCl solution for 24 h before being rinsed with deionised water. Each IEM was inserted vertically into the soil such that the shallow end of the IEM was just below the soil surface. Four such tags were inserted throughout each sector to account for soil heterogeneity. The tags were withdrawn from soil following a 14-d period, after which they were immediately replaced by a charged IEM tag. This process was repeated fortnightly from 25 February 2016 through 16 January 2017 with a gap in data in August 2016. IEM were stored at 4°C for no more than 24 h prior to analysis. Soil and organic matter were removed using a brush and deionised water. NH_4^+ and NO_3^- were extracted by shaking each IEM in a 0.05 *M* HCl solution for 1 h. The extracts were filtered through 0.22 µm nylon filters before being analysed colorimetrically using a SmartChem 200 Discrete Analyser (Westco Scientific Instruments, Inc., Brookfield, United States).

3.2.2 Potential net nitrogen transformations and soil carbon and nitrogen

Seven soil cores (10 mm diameter × 50 mm depth) were taken from each sector 38 d following urea application. The gaps in the soil were filled with bulk site soil following the removal of soil samples. Cored locations were marked with a flag to prevent sampling from that area of the plot again. Cores were bulked and chilled for 2 h before being processed. In order to compare inherent rates under uniform temperature and soil moisture conditions, soil

samples were partially air dried overnight and handpicked to remove coarse organic matter. Following this, all incubations were performed at uniform soil water content and in a dark environment at room temperature. The initial pool size of NH_4^+ and NO_3^- was assayed by extracting a 5 g subsample using 2 *M* KCl within 24-48 h of collection. This solution was centrifuged, after which the supernatant was filtered through 0.22 µm nylon filters and analysed colorimetrically on a discrete analyser. Two 10 g subsamples were adjusted to 60% field capacity using deionised water. To obtain N-mineralisation rates in N-limited and N-replete conditions, one sample was amended with 4 mg urea, while the other was provided no amendment. Soil samples were incubated in sealed glass jars at 30°C for 28 d. Jars were opened weekly to prevent hypoxia. Incubated soil samples were then extracted and assayed exactly as the initial subsample. Potential net nitrification, potential net ammonification and potential net mineralization rates were measured as the change in 2 *M* KCl-extractable NH_4^+, NO_3^- and mineral N (NH_4^+ + NO_3^-) after incubation of soils. The capacity for N mineralisation is tightly associated with the organic matter present in soils estimated by soil C content (O'Brien and Stout 1978). As such, these transformations are expressed on elemental weight basis per gram soil C (ug N g^{-1} C). Total soil C and N was determined by grinding the remaining bulked, air dried sample in a Mixer Mill MM200 ball mill (Retsch, Newtown, USA) and analysed for C and N using a Perkin-Elmer 2400 Series II Elemental Analyser (Perkin Elmer, Melbourne, Australia).

3.2.2 Statistical analysis

A linear mixed effects model with repeated measures was used to test the impact of date, CO_2 treatment and irrigation on IEM-available NH_4^+ and NO_3^- using the LME4 package (Bates et al. 2015) in R version 3.5.0 (R Core Team 2018). CO_2 level, irrigation, date as well as CO_2 × irrigation and CO_2 × date interaction terms were included as fixed factors. Winter irrigation treatments were excluded from this analysis given irrigation volumes were uniform for all

treatments. The model containing the CO_2 × date × irrigation interaction as well as the model containing the date × irrigation interaction were not included in the analysis for NH_4^+ or NO_3^- due to AICΔ > 2.0. As a random effect, I included plot number in a random intercept model. I performed model selection using the Likelihood Ratio Test, in which I used ANOVA to compare a model containing all terms with a model excluding one term of interest. This process was repeated for all fixed factors in the first model. Only terms where $P \leq 0.05$ were included in the final model. I then performed *post hoc* pairwise comparisons using the R package 'emmeans' (Lenth 2018). Where necessary, data were transformed to avoid deviations from homoscedasticity and normality. This same method was used to test the impact of CO_2, irrigation and their interaction on soil C, N, C:N, N transformations and WFPS.

3.3 Results

3.3.1 Soil water content

Soil water content varied between seasons, with a tendency for lower WFPS in summer compared to other seasons (Fig. 3.1). There was high variability within spring sampling periods compared to the narrow range of values at other times. There was a tendency for greater WFPS under eCO_2, though WFPS under 550 μmol CO_2 mol^{-1} was never higher than

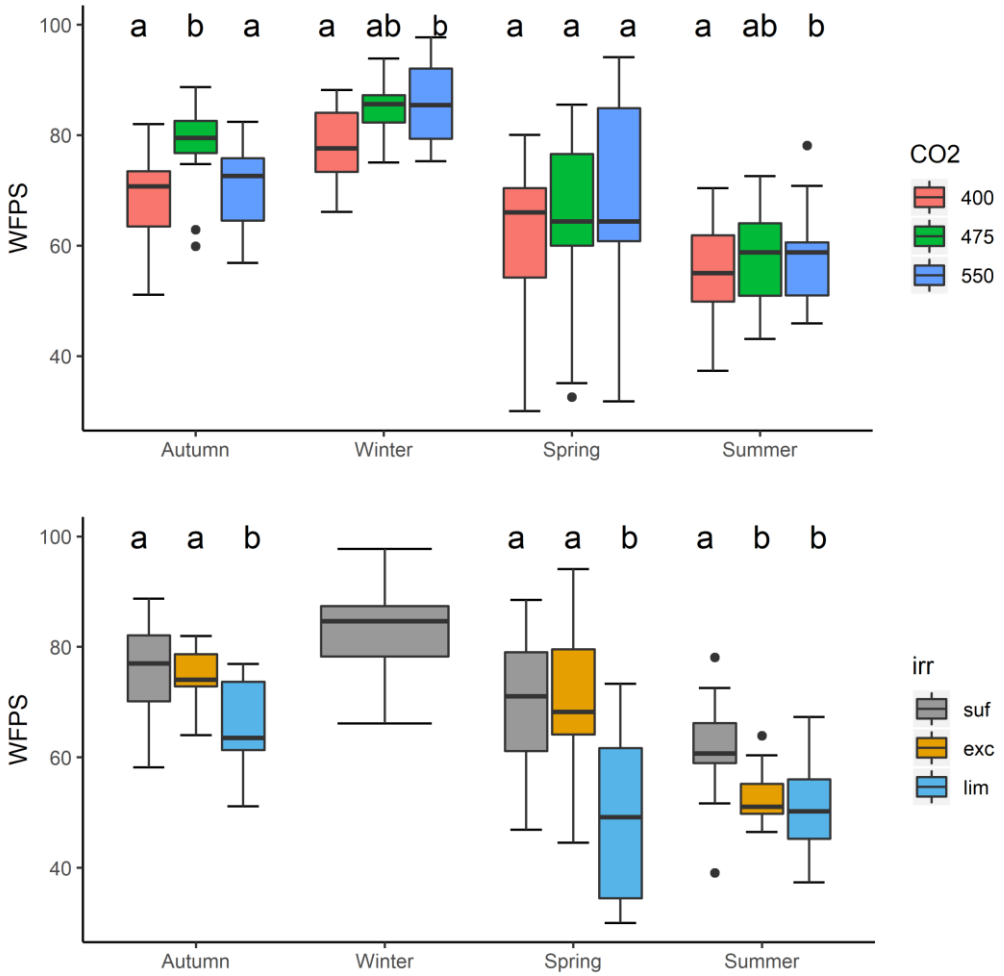

Figure 3.1. Percent water-filled pore space (WFPS; mean ± SEM) for each season in 2016. Data were measured in soils under three CO_2 levels (400, 475, 550 μmol CO_2 mol^{-1}; top) and three irrigation treatments (*sufficient, limit, excess*; bottom). Note that all treatments received *sufficient* in winter. Significant differences were determined using pairwise comparison of a linear mixed effects model containing CO_2 × date interaction term as a fixed factor with plot number as a random factor. Those means with the same letter within a date are not significantly different ($P > 0.05$).

457 WFPS under 475 μmol CO_2 mol^{-1} (Fig. 3.1). Furthermore, there was no incremental change
458 in WFPS with [CO_2]. WFPS tended to be lower in the *limit* irrigation treatment than *sufficient*
459 and *excess* (Fig. 3.1). WFPS in the *excess* treatment was not higher than the other treatments
460 in any season.

461 *3.3.4 Soil N availability*

462 Inorganic N availability varied substantially over time, as free levels of NH_4^+ and NO_3^- were
463 highly responsive to urea application (Fig. 3.2). These peaks were ephemeral, with soil
464 mineral N availability typically decreasing to comparatively low levels within two to four
465 weeks of urea application. The N contained in the NO_3^- pool was more than 3 times higher
466 than the N contained in NH_4^+ (Figs. 3.2 and 3.3), suggesting that when made available, NH_4^+
467 was rapidly oxidized to NO_3^-. This also demonstrates that the mineral N pool was comprised
468 primarily of NO_3^-. Consequently, eCO_2 effectively lowered NO_3^- levels and therefore also
469 lowered the total mineral N pool (Fig. 3.2). NO_3^- peaks were highest in winter and early
470 spring compared to those at other dates ($P < 0.001$), likely due to low plant N demand and
471 lower temperatures. Soil NO_3^- availability was generally highest in plots exposed to 400 μmol
472 CO_2 mol^{-1} and lowest in plots exposed to 550 μmol CO_2 mol^{-1}, with plots at 475 μmol CO_2
473 mol^{-1} being intermediate. This pattern was particularly noticeable in the peaks following urea
474 application (Fig. 3.2). The CO_2- effect was strongest in spring and summer when NO_3^- levels
475 in plots at 475 and 550 μmol CO_2 mol^{-1} were approximately half that in plots exposed to 400
476 μmol CO_2 mol^{-1} (Fig. 3.2). Statistically, soil NO_3^- availability was significantly lower in 550
477 μmol CO_2 mol^{-1} plots than in plots at 400 μmol CO_2 mol^{-1} on 11 out of 18 individual
478 occasions (Fig. 3.2). When expressed as cumulative availability, an important measure of the
479 total availability of mineral N during a growing season, it is obvious that increasing the [CO_2]
480 reduced plant access to soil N (Fig. 3.3). By the beginning of winter, cumulative mineral N
481 had declined 45% under 550 μmol CO_2 mol^{-1} compared to 400 μmol CO_2 mol^{-1} (Fig. 3.3). By

the end of winter, cumulative mineral N was 25% lower under 475 μmol CO_2 mol^{-1} and 41% lower under 550 μmol CO_2 mol^{-1} compared to 400 μmol CO_2 mol^{-1} (Fig. 3.3). It is clear declines in mineral N availability were non-linear with [CO_2] given an increase of 75 μmol CO_2 mol^{-1} from 400 μmol CO_2 mol^{-1} yielded a large decline in mineral N, yet an addition 75 μmol CO_2 mol^{-1} from 475 μmol CO_2 mol^{-1} was relatively minor (Fig. 3.3).

Compared to NO_3^-, NH_4^+ availability was affected only modestly by [CO_2], an effect which was present in just a few dates (Fig. 3.2 and Table 3.1). NH_4^+ was lower under 550 μmol CO_2 mol^{-1} than any other CO_2 level only in Oct 31 ($P < 0.04$) and Nov 14 ($P < 0.03$). On 16 Jan both 475 and 550 μmol CO_2 mol^{-1} were lower than 400 μmol CO_2 mol^{-1} ($P < 0.03$). No similar effect was present in the cumulative measure of NH_4^+ as the availability of this ion remained similar between CO_2 levels (Fig. 3.3; $P > 0.5$). NH_4^+ comprised approximately 10% of the cumulative mineral N pool by the end of the experiment as the majority of the pool was NO_3^- (Fig. 3.3).

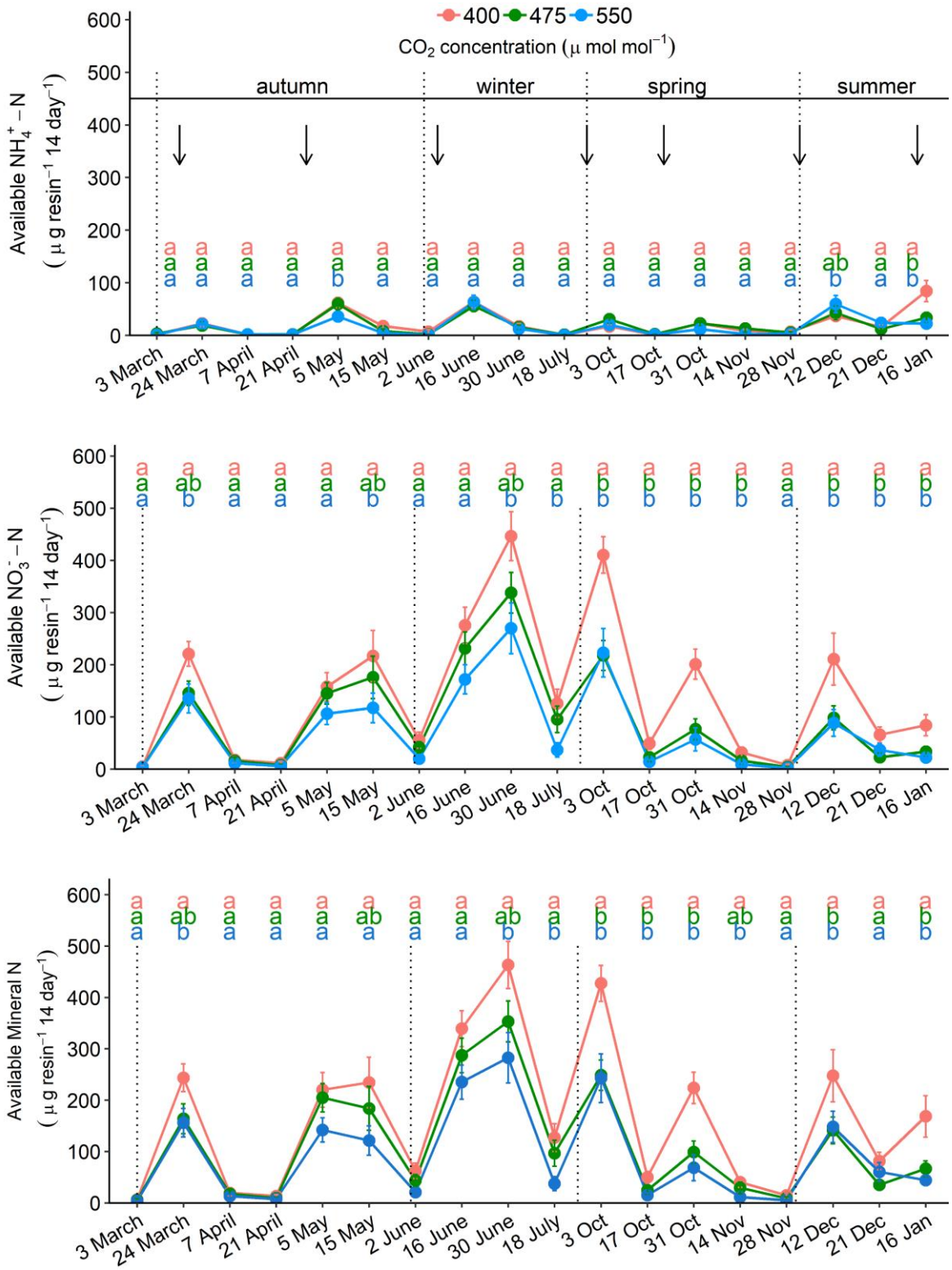

Figure 3.2. Impact of CO_2 on ion-exchange membrane-available ammonium-N (NH_4^+; top), nitrate-N (NO_3^-; middle) and mineral N (bottom) for each sampling date (mean ± SEM). Measurements were collected from sectors exposed to three levels of CO_2 (400, 475 and 550 µmol CO_2 mol^{-1}) from 3 March 2016 through 16 January 2017. Those means with the same letter within a date are not significantly different ($P > 0.05$). Dotted lines indicate season bounds. Arrows indicate an aboveground biomass harvest and urea application.

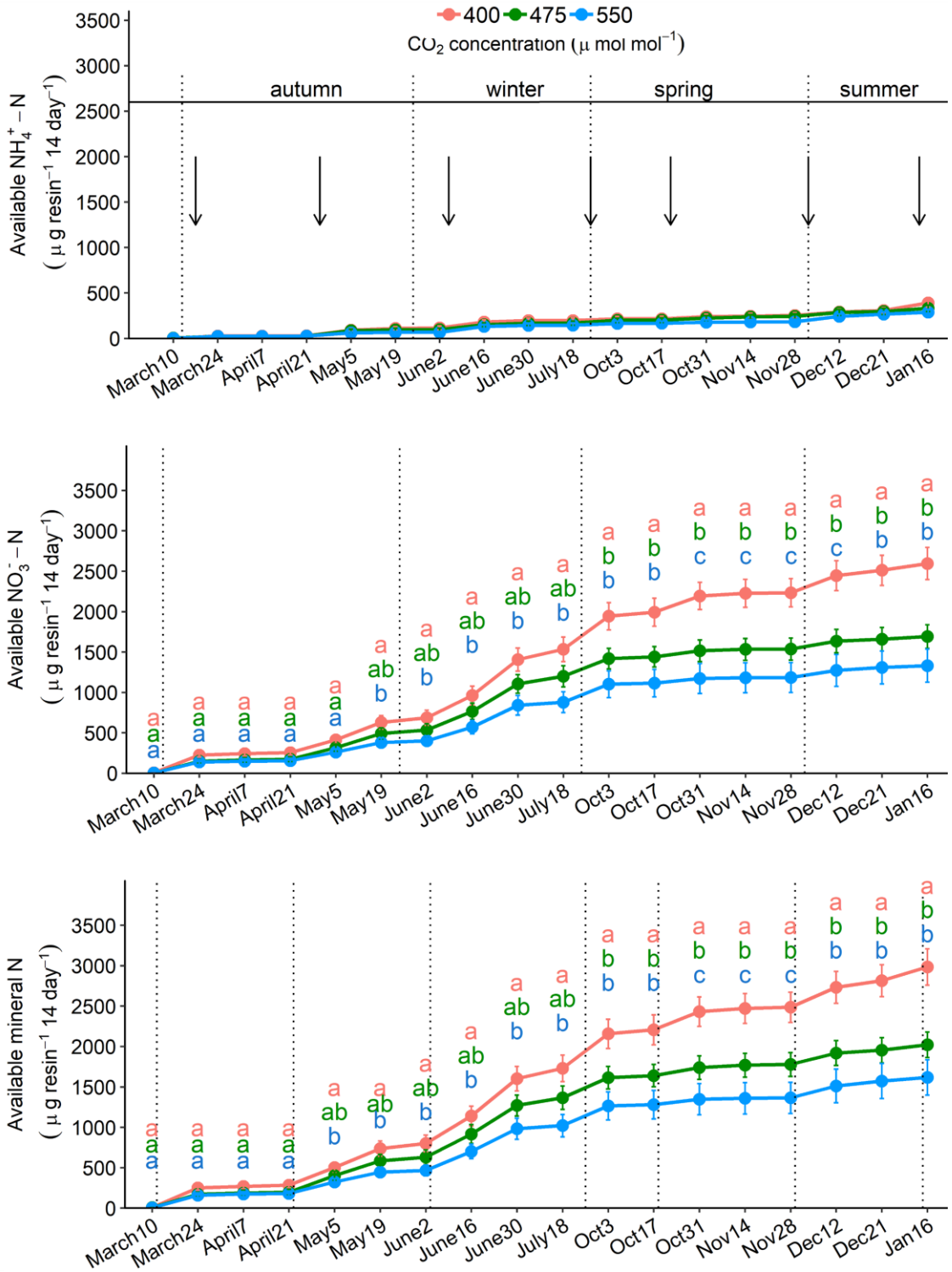

Figure 3.3. Impact of CO_2 on cumulative ion-exchange membrane-available ammonium-N (top), nitrate-N (middle) and total available mineral N (bottom) calculated for each date (mean ± SEM). Measurements were collected from sectors exposed to 3 levels of CO_2 (400, 475 and 550 µmol CO_2 mol^{-1}) from 3 March 2016 through 16 January 2017. Those means with the same letter within a date are not significantly different ($P > 0.05$). No significant impact of CO_2 on NH_4^+ was present. Dotted lines indicate season bounds. Arrows indicate an aboveground biomass harvest and urea application.

Compared to NO_3^-, NH_4^+ availability was affected only modestly by $[CO_2]$, an effect which was present in just a few dates (Fig. 3.2 and Table 3.1). NH_4^+ was lower under 550 μmol CO_2 mol^{-1} than any other CO_2 level only in Oct 31 ($P < 0.04$) and Nov 14 ($P < 0.03$). On 16 Jan both 475 and 550 μmol CO_2 mol^{-1} were lower than 400 μmol CO_2 mol^{-1} ($P < 0.03$). No similar effect was present in the cumulative measure of NH_4^+ as the availability of this ion remained similar between CO_2 levels (Fig. 3.3; $P > 0.5$). NH_4^+ comprised approximately 10% of the cumulative mineral N pool by the end of the experiment as the majority of the pool was NO_3^- (Fig. 3.3).

Irrigation had little effect on mineral N availability throughout the experiment (Table 3.1). There was a minor effect of irrigation on NH_4^+ availability only on several dates but I identified no pattern for these differences. NH_4^+ was lower in *excess* sectors relative to *limit* in 3 Oct and 17 Oct. Several months later in January, NH_4^+ in *excess* sectors was higher than both *limit* and *sufficient* ($P < 0.02$). There was no effect of irrigation on NO_3^- availability at any time nor was there a CO_2 × irrigation interaction. Importantly, this indicates that the CO_2 effects were robust across the irrigation treatments.

Table 3.1. Results of model selection using the Likelihood Ratio Test. ANOVA was used to compare a linear mixed effects model which contained all terms to the same model without the term of interest. Bold text indicates a significant term (P-value ≤ 0.05)

Response var.	Fixed Factor	Chi sq.	df	P-value
Ammonium (NH_4^+)	Date	808	17	**< 0.001**
	CO_2	6.9	2	**0.03**
	Irrigation	10.1	2	**< 0.01**
	CO_2 × Date	49.3	34	**0.04**
	Irrigation × CO_2	7.3	4	0.1
Nitrate (NO_3^-)	Date	990	17	**< 0.001**
	CO_2	21.7	2	**< 0.001**
	Irrigation	4.5	2	0.1
	CO_2 × Date	47.9	34	**0.05**
	Irrigation × CO_2	1.4	4	0.8

3.3.2 Soil N mineralisation potential

Potential net mineralization rates of soils from TasFACE2 were measured under controlled moisture and temperature conditions in the laboratory (Table 3.2). Net ammonification rates were negative for all treatments, while net nitrification rates were both positive and substantially higher than net ammonification rates (Table 3.2). Thus, in this soil NH_4^+ was rapidly oxidized to NO_3^-, an effect which persisted in both autumn and winter. The high relative rate of net nitrification relative to net ammonification was even more pronounced in the soils with urea addition. In soils amended with urea, net nitrification rates were 360% higher than in soils without this addition (Table 3.2). However, ammonification rates were similar in the presence or absence of urea (Table 3.2), further emphasizing the dominance of nitrification in this system. Given the high nitrification rates, it is unsurprising available NO_3^- was substantially higher than NH_4^+ (Figs. 3.2 and 3.3). There were no differences in potential net mineralisation between CO_2 levels ($P > 0.1$) or irrigation ($P > 0.1$).

Table 3.2 Potential net ammonification and nitrification rates (mean and SEM) in soils from TasFACE2 in autumn and winter, with and without urea amendment**.** There was no difference in net ammonification between the control and soils with a urea addition ($P > 0.1$) but there was a significant effect of urea addition on net nitrification in these incubations ($P < 0.001$). There were no differences in potential net mineralisation between CO_2 levels ($P > 0.1$) or irrigation ($P > 0.1$).

		Net ammonification Δ NH_4^+-N (μg N/g C/ 28 d)				**Net nitrification** Δ NO_3^--N (μg N/g C/ 28 d)			
	[CO_2]	Control		+ Urea		Control		+ Urea	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Aut.	400	-247	60	-200	57	1 627	572	9 243	687
	475	-206	56	-144	57	1 441	687	9 286	926
	550	-182	61	-115	65	1 656	744	8 945	1 289
Win.	400	-238	53	-190	63	2 492	639	9 748	621
	475	-289	120	-207	130	2 510	621	9 319	996
	550	-205	48	-127	49	2 516	464	10 085	693

3.3.3 Soil C and N

The C:N of soil organic matter can influence mineralisation rates as low N content can limit microbial activity. I found no significant impact of CO_2 level, irrigation or their interaction on soil C:N in autumn or winter ($P > 0.1$; Fig. 3.4). This agrees with the incubation study which found no difference in potential mineralisation rates in a controlled environment (Table 3.2). Total soil C was lowest under 550 μmol CO_2 mol^{-1}, was intermediate in 475 μmol CO_2 mol^{-1} and highest under 400 μmol CO_2 mol^{-1} (Table 3.3). Total soil N followed this same pattern in autumn, though only 550 was lower than 400 μmol CO_2 mol^{-1} in winter.

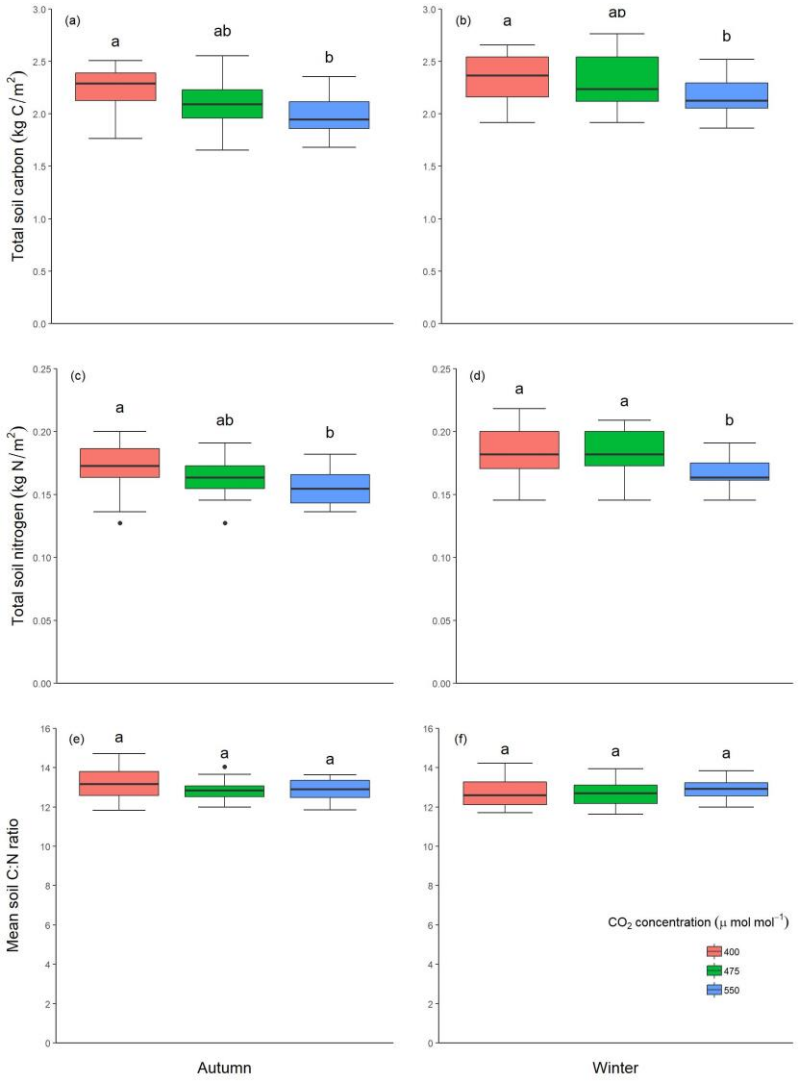

Figure 3.4. Total soil C (a and b), total soil N (c and d) and soil C:N (e and f) measured in TasFACE2 in autumn and winter 2016. Those boxes with the same letter for each date and element are not significantly different ($P > 0.05$).

3.4 Discussion

The findings presented here demonstrate that exposure of this fertilised pasture to eCO_2 lowered available mineral N within the first growing season, nearly entirely due to declines in the NO_3^- pool. Available NH_4^+ was rapidly oxidised to NO_3^- (Table 3.2), resulting in a substantially larger NO_3^- pool relative to NH_4^+ (Fig. 3.2). The potential net mineralisation rates measured in laboratory incubations were similar among CO_2 levels (Table 3.2), demonstrating that differences in mineral N were not due to a CO_2-effect on the rate of mineral N input from microbial activity. On most sample dates, N availability was lower in plots exposed to eCO_2 than in control plots, but there was little difference between the two eCO_2 levels (Fig. 3.2). However, when expressed cumulatively, it is clear that mineral N reduced sequentially from 400, to 475 and to 550 μmol CO_2 mol^{-1} (Fig. 3.3). This decline was non-linear, suggesting the effects driving this decline may have a similar response to $[CO_2]$. Importantly, declines in mineral N availability were affected by $[CO_2]$ but were independent of irrigation and CO_2 × irrigation, indicating that the CO_2-effect on plant production, plant N requirements and belowground C input drove these changes. Therefore, in this system, the CO_2 effect on soil N availability was independent of CO_2 effects on soil moisture.

One possible mechanism for the decline in mineral N in this system is the stimulation of plant growth under eCO_2. Increased biomass production under eCO_2 leads to immobilisation of mineral N in plant tissues and soil organic matter pools reducing plant and microbial access to N through Progressive Nitrogen Limitation (Luo et al. 2004). Increased transport and storage of N from soil mineral pools into plant biomass has been found in other pastures (Hu et al. 2001, Gill et al. 2002a, Reich et al. 2006a, Newton et al. 2010). In TasFACE2, plant production was stimulated under eCO_2, generally resulting in higher plant N (Fig. S1). Interestingly, this was entirely due to greater root production under eCO_2, an effect which

was strong enough to compensate for declines in aboveground biomass production with [CO_2] (Fig. S1). In fact, the largest gap in cumulative mineral N availability between [CO_2] levels developed in spring (Fig. 3.3), the season with the most prominent increase in root production under 550 μmol CO_2 mol^{-1} (Fig. S1). In addition to immobilising more N, the CO_2-effect on root production undoubtedly provided plants grown under 550 μmol CO_2 mol^{-1} more root surface area for N uptake than plants grown in ambient CO_2 (Finzi et al. 2007). However, stimulation of root production was substantial in 550 μmol CO_2 mol^{-1} only, with little difference in root production between 475 and 400 μmol CO_2 mol^{-1} (Fig. S1). Given mineral N availability was reduced incrementally with [CO_2], immobilisation of N in roots likely contributed to mineral N declines but by itself cannot explain the patterns observed. Under eCO_2, N immobilised in microbial biomass has been shown to increase in some grasslands and is associated with declines in N availability (Hungate et al. 1997c, Pleijel et al. 1998, de Graaff et al. 2006, Sun et al. 2017). In TasFACE2, declines in total soil N demonstrate that no additional N was sequestered in soil under eCO_2 (Fig. 3.4). Although this is not a direct measure of microbial biomass N, it does suggest that increased immobilisation of N in the microbial pool is an unlikely explanation for the CO_2-driven decline in mineral N observed.

The loss of mineral N under eCO_2 may have been due in part loss of N through gaseous emissions. Denitrification can be a major source of NO, N_2O and N_2 in soils with high available NO_3^-, organic matter and WFPS between 60-90% (Firestone and Davidson 1989a, Henry et al. 2008). Many studies have attributed high rates of gaseous N loss under eCO_2 to plant-mediated stimulation of denitrification via organic matter input and increased soil water content (Arnone and Bohlen 1998, Kammann et al. 2008, van Groenigen et al. 2011, Martins et al. 2016). WFPS in TasFACE2 often exceeded 60% but was largely unaffected by [CO_2] (Fig. 3.1). Despite only a minor CO_2-effect on WFPS, greater C input under eCO_2 may have

stimulated heterotrophic denitrifier activity as found in other pastures (Luo et al. 1999). Gaseous N emissions from nitrification are generally low relative to denitrification (Mathieu et al. 2006), but can be substantial following the application of ammonium yielding fertiliser (Tortoso and Hutchinson 1990, Bremner 1997, Zhu et al. 2013b). However, it is unclear how nitrification might have caused an incremental decline in mineral N availability with [CO_2]. Based on our laboratory incubations, there were high nitrification rates but no indication of a CO_2-effect on nitrification (Table 3.2). A meta-analysis of similar studies demonstrated net nitrification is typically increased under eCO_2, but gross nitrification rates can be either suppressed or stimulated (Barnard et al. 2005). As I found no indication of a CO_2-effect on nitrification, this is an unlikely source of mineral N loss. Leaching is another possible explanation for the loss of mineral N in this system. NO_3^- is vulnerable to leaching as soil particles are negatively charged and bind only to cations such as NH_4^+. Thus, most of the mineral N pool in this grassland was susceptible to transport in soil solution. However, a CO_2-induced stimulation of leaching was unlikely here due to the accumulation of moisture at 30 cm where the sandy loam horizon intersects medium to heavy clay. Similar agricultural systems have demonstrated strongly reduced NO_3^- leaching through clayey soils compared to sandy loam (Hoffmann and Johnsson 1999, Beaudoin et al. 2005). As the rooting depth here is approximately 40 cm, it is likely most of the NO_3^- at this junction would be immobilised in plant biomass. It is expected that *sufficient* and *excess* did not vary consistently given any soil water exceeding field capacity would be quickly drained through the soil. The contribution of each of these pathways to mineral N losses here cannot be ascertained as the methods required to do so are beyond the scope of this study. The contribution of both nitrification and denitrification could be quantified using isotopic analysis of NO, N_2O and N_2 from soils (Kool et al. 2010). Mineral N losses via leaching could be estimated using lysimeters or a similar method of soil solution capture and analysis (Scholefield et al. 1993).

N mineralisation can alter the size of soil N pools through the regulation of new mineral N inputs from the breakdown of organic N (Binkley and Hart 1989). In an incubation of soils from TasFACE2, potential net N mineralisation rates were unaffected by CO_2 level (Table 3.2). A similar pattern likely was present in field conditions, suggesting the input of mineral N was similar between CO_2 treatments. Thus, differences in mineral N between CO_2 levels were likely due to loss of N through immobilisation, gaseous loss or leaching rather than a change in N input. It is difficult to generalise the effect of [CO_2] on N mineralisation rates as some pastures show [CO_2] can reduce (Hungate et al. 1999, Gill et al. 2006, Reich et al. 2006a), increase (Cheng et al. 2001, McKinley et al. 2009, Björsne et al. 2014) or have no effect. Gill et al. (2002a) found N mineralisation rates declined with [CO_2] due to changes in litter chemistry. However, in that study the differences in mineralisation rates between 400 μmol CO_2 mol^{-1} and 550 μmol CO_2 mol^{-1} were minor relative to changes below 400 μmol CO_2 mol^{-1}. In TasFACE2, the C:N ratio of soils did not vary between CO_2 levels (Fig. 3.4); thus, it is unsurprising mineralisation similarly was unaffected by CO_2 treatments. eCO_2 tends to decrease the litter quality of plant tissues, which can lead to higher soil C:N and greater microbial immobilisation (Hu et al. 2001, de Graaff et al. 2006). However, changes in soil C:N under eCO_2 typically occur only following years of fumigation (Smith 2004, de Graaff et al. 2006). It is difficult to predict how soil C:N may change over time in TasFACE2 based on current management practices of the site. Aboveground biomass inputs are removed through routine harvest, thus organic matter inputs are limited to root mortality and root exudation. Therefore, it is likely that a CO_2-effect on soil C:N may take many years to develop.

Soil C and N were reduced under eCO_2, with up to 7% loss within the first growing season with no associated change in soil C:N (Fig. 3.4). Mineral N levels provide little explanation for these losses given soil N was measured over 5 weeks following urea application and was likely depleted of urea-N (Fig. 3.2). Rather, the loss of C and N in response to eCO_2 over a

short fumigation period is indicative of accelerated C and N cycling via microbial 'priming' (Cheng and Johnson 1998, Shahzad et al. 2015a, Huo et al. 2017). Following addition of labile C, soil microbial growth is increased and consequently microbial C and N demand (Kuzyakov 2002a, Zhu et al. 2014). To meet microbial nutritional requirements, decomposition of soil organic matter is stimulated, resulting in loss of soil C and N through mining of recalcitrant pools (Kuzyakov 2002a, Zhu et al. 2014). This effect is common under eCO_2 and is attributed to increased exudation rates (Cheng and Johnson 1998, van Groenigen et al. 2014, Shahzad et al. 2015a) which stimulate the production of extracellular enzymes (Brzostek et al. 2013) and fungal exploration and uptake of organic C and N (Hodge and Fitter 2010, Cheng et al. 2012). Given there was substantially more photosynthate supply and root biomass under eCO_2 in TasFACE2, it is likely rhizodeposition increased here as was observed in similar systems (Pendall et al. 2004, de Graaff et al. 2006). This also explains why the decline in soil C and N occurred under 550 μmol CO_2 mol^{-1}, which had substantially greater root production than other CO_2 levels (Fig. S1). Interestingly, I found no indication of increased N mineralisation under eCO_2 in our laboratory incubations despite measured declines in soil C and N. This may be partly due to the absence of root exudation in the incubations, which can alter mineralisation and inorganic N availability even over short periods (Hamilton et al. 2008). Therefore, the potential net N mineralisation rates provide a useful tool for assessing N cycling but may not fully reveal the impacts of [CO_2] on mineralisation rates. Measurement of *in situ* C and N mineralisation rates might help to provide explanations for the observed declines in soil C and N.

In conclusion, this study demonstrates that soil mineral N availability over a growing season was reduced incrementally by increasing [CO_2] in a well-fertilised, irrigated temperate pasture. The CO_2-effect on N cycling was independent of irrigation treatments, demonstrating the importance of production on N cycling here. Immobilisation of mineral N in plant

biomass contributed to lower N availability under eCO_2 as was expected based on Progressive Nitrogen Limitation theory (Luo et al. 2004). However, the decline in mineral N cannot be explained by increased storage of N in plant biomass entirely as plant N content increased under eCO_2 but did not increase further with higher [CO_2]. I determined that nitrification and denitrification are likely pathways contributing to gaseous N losses from this system. Denitrification in particular may have been stimulated by the indirect impact of eCO_2 on organic C supply to denitrifiers. Investigation of the CO_2-effect on N emissions might help to assess the loss of soil mineral N as well as quantify the emission of N_2O, a potent greenhouse gas, from this grassland. The results of such an investigation are presented in Chapter 4 of this thesis. Although I determined the indirect effect of CO_2 on soil water content was inadequate to increase N losses via leaching, measurement of leachate would ascertain the role of this pathway in this system.

4 EFFECT OF ELEVATED CARBON DIOXIDE ON NITROUS OXIDE EMISSIONS

4.1 Introduction

The concentration of nitrous oxide (N_2O) in the atmosphere has increased from 270 ± 0.7 ppb at pre-industrial levels to 324.2 ± 0.1 ppb, a 20% increase since pre-industrial levels (Meure et al. 2006). The concentration of N_2O has increased at an average rate of 0.75 ppb yr^{-1} and is projected to continue rising (Ciais et al. 2013). N_2O is a gas of great importance to climate change due to its high contribution to positive radiation forcing, long atmospheric lifespan of 118-131 years (Fleming et al. 2011) and ozone-depleting properties (Ravishankara et al. 2009). N_2O is responsible for the third largest radiative forcing of all greenhouse gases, after carbon dioxide (CO_2) and methane but has 298 times the global warming potential of CO_2 over a 100-year period (IPCC 2014). Soils are the largest source of N_2O, with 4.1 Tg N_2O-N yr^{-1} emitted from agricultural land and 6.6 Tg N_2O-N yr^{-1} from soils with natural vegetation, collectively yielding a global total of 11.0 Tg N yr^{-1} (Thomson et al. 2012).

Most N_2O soil emissions are produced through nitrification and subsequent denitrification. Nitrification is an aerobic process performed in two steps where ammonia is oxidized to nitrite and finally nitrate (NO_3^-), predominantly by ammonia oxidizing bacteria and archaea followed by nitrite oxidising bacteria, or in a single step by commamox bacteria (Beeckman et al. 2018). Fungi have also been found to carry out nitrification and are capable of oxidizing both ammonium (NH_4^+) and organic nitrogen (N) to NO_3^- (Schimel et al. 1984, Laughlin et al. 2008, Maeda et al. 2015). Soil NO_3^- is then acquired and immobilized by plants but can also leach or be denitrified in anaerobic soils (Bateman and Baggs 2005). Nitrification yields N_2O as a by-product following oxidation of NH_4^+ while denitrification produces N_2O through the incomplete reduction of NO_3^- to dinitrogen (N_2). As nitrification is an aerobic process and denitrification is anaerobic, soil moisture strongly affects which N transformation pathway

predominates at any particular time. When soil water content is between 60 and 90% water-filled pore space (WFPS), incomplete denitrification yields a high ratio of N_2O:N_2 end products, while above 90% WFPS NO_3^- and nitrite are generally completely reduced to N_2 (Davidson et al. 2000). Therefore, denitrification rates rise as soil water content increases, though the production of N_2O diminishes at very high soil water content.

Elevated CO_2 (eCO_2) may increase N_2O emissions by altering the factors underlying N transformations such as soil moisture, belowground organic matter inputs and available N. Under eCO_2, plant stomatal conductance and transpiration are both reduced, which increases plant water-use efficiency and increases soil water content (Long et al. 2004, Nelson et al. 2004, Martins et al. 2016). Increased soil water content may have a stimulatory impact on heterotrophic denitrification by reducing soil oxygen availability (Smith and Tiedje 1979), subsequently increasing the occurrence of anaerobic sites in soil and N_2O emissions (Khalil et al. 2004). Plant photosynthesis increases with [CO_2] (Leakey et al. 2009), which stimulates belowground C input through increased litter supply (de Graaff et al. 2006) and rhizosphere deposition (Iversen et al. 2008). Increased belowground C input may stimulate N_2O production via denitrification as heterotrophs use organic matter as an energy supply (Firestone and Davidson 1989a, Henry et al. 2008). eCO_2 may also alter soil N availability, which can regulate N transformations and consequently N_2O emissions. Several studies have demonstrated soil N availability, NO_3^- in particular, is typically lower under eCO_2 due in part to the CO_2-effect on plant productivity and N immobilization (Hovenden et al. 2008, Dijkstra et al. 2010b). However, plant immobilization cannot explain declines in N alone because the same effect was present at times when primary production was unaffected by eCO_2 (Hovenden et al. 2008). This suggests CO_2-stimulated denitrification could increase N_2O losses from soils, thereby contributing to the observed decline in soil N availability. Although

the response of N_2O emissions to eCO_2 varies, most studies report an increase in emissions attributed to plant-mediated shifts in soil water content and organic matter supply (Arnone and Bohlen 1998, Kammann et al. 2008, van Groenigen et al. 2011, Martins et al. 2016). However, a body of studies report no significant CO_2-effect on N_2O emissions due to low soil N availability (Ambus and Robertson 1999, Mosier et al. 2002, Baggs et al. 2003b, Dijkstra et al. 2013, Sun et al. 2017). In some studies, plant immobilization of soil N reduced nitrification and denitrification rates such that N_2O emissions were reduced under eCO_2 (Hungate et al. 1997c, Pleijel et al. 1998, Kettunen et al. 2007), while others reported both low N and water availability (Dijkstra et al. 2013). Most studies indicate a positive association between eCO_2 and N_2O emissions in grasslands, but competing influences make generalising the response of N_2O to eCO_2 difficult. As soil mineral N availability can drive N_2O emissions, other influences driven by eCO_2 such as belowground C input can be dwarfed in the presence of high soil N. Measurement of N_2O emissions in periods of both low and high mineral N availability may help to determine whether belowground C inputs in a relatively low soil mineral N environment can alter N_2O emissions. A greater understanding of the mechanisms underlying the N_2O response to eCO_2 and soil N is essential to accurately predict the contribution of grasslands to climate change under future atmospheric conditions.

Here, I aim to determine the impact of eCO_2 on N_2O fluxes in a *Lolium perenne*-dominated grassland. I measured N_2O emissions at periods of different soil mineral N availability to determine if the CO_2-effect on emissions varied with soil N. I hypothesised that mineral N availability would be the main factor influencing N_2O emissions in this grassland. If this is the case, then the CO_2-effect mediated by plant N immobilisation described in Chapter 3 also strongly influences N_2O emissions in this grassland given the strong effect documented. I applied three levels of CO_2 (400, 475 and 550 μmol CO_2 mol^{-1}) to measure the CO_2-effect

size on N_2O emissions and to assess whether that effect increases with CO_2 concentration ($[CO_2]$). As cumulative mineral N availability declined with $[CO_2]$, I hypothesised that N_2O emissions similarly would change with $[CO_2]$. Further, by excluding rainfall in combination with irrigation, I could determine the influence of water supply and $[CO_2]$ on N_2O emissions. Labile soil NO_3^- and NH_4^+ were measured to investigate the effects of $[CO_2]$ on soil mineral N pools and explore the linkages between soil mineral N availability and N_2O emissions. Furthermore, I measured N_2O emissions for 7 d following urea application to determine the influence of N availability on peak N_2O emissions. I measured emissions again well after peak emissions to assess whether the CO_2-effect changed as mineral N availability declined. N_2O emissions were also measured after peak emissions in two seasons to assess whether the N_2O response varied between seasons. Plant above- and belowground biomass production was measured and analysed for C and N to assess the relationship between plant immobilization of N and $[CO_2]$. As most FACE experiments are limited to two CO_2 levels, this experiment provides insight into the possible shape of the CO_2-response curve of N_2O emissions, potentially improving our ability to predict these processes in the future.

4.2 Methods

4.2.1 Gas sampling and analysis

I measured N_2O fluxes from each sector using static chambers (Dijkstra et al. 2013). The static chambers (diameter 10 cm, height 20 cm) were covered in reflective Mylar and equipped with a nylon tube 'pigtail' (length 8 cm) connected to a narrow outlet for passive pressure compensation. Two adjustable latches on the chamber were used to form a tight seal with a rubber gasket fitted to the soil collar. I inserted one collar (PVC; diameter 10 cm, height 10 cm) 8 cm into the soil in each sector ~30 d prior to the first measurement. The chamber headspace was sampled sequentially over a 1-hr period at 0, 20, 40 and 60 min

between 10 a.m. and 3 p.m. Each chamber contained an iButton (Maxim Integrated, San Jose, CA, USA) temperature probe that recorded internal chamber temperature on 1-min intervals. A 25-ml headspace sample was collected using a gas-tight syringe (SGE, 25MDR-LL-GT, Melbourne Australia) and stored in an evacuated 12-ml Exetainer (Labco, High Wycombe, Buckinghamshire, UK). Samples were analysed within one month on an Agilent 7890A gas chromatograph fitted with a Gerstel MPS2-XT auto sampler. The gas chromatograph was equipped with two channels leading to micro-electron capture detector (μ-ECD) and flame ionisation detector (FID) for the analysis of CO_2 and N_2O. Fluxes were calculated using the slope of the linear regression of the gas concentration over time. Measurements were recorded for N_2O if the regression r^2 value was above 0.8 for N_2O as well as CO_2. N_2O flux was expressed on elemental weight basis (μg N_2O-N m^{-2} h^{-1}) after being corrected for chamber air temperature, atmospheric pressure, chamber volume and surface area using the equations in Scheer et al. (2014).

4.2.2 Seven-day N_2O campaign

N_2O emissions are often highest for several days immediately following the application of nitrogenous fertiliser due to high N availability (Hungate et al. 1997c). Emissions during this period can comprise a large proportion of the overall N_2O released from managed grasslands (Hungate et al. 1997c, Baggs et al. 2003b). Given the high sampling frequency required to accurately quantify this peak, this campaign was focused on measuring the CO_2-effect on N_2O emissions rather than the watering effect on emissions. Thus, only sectors receiving *sufficient* water were measured. Daily gas sampling began 24-h following urea application on 17 October 2017 and continued until 23 October 2017. Gas samples were collected in two complete blocks. Each sector was irrigated on day 1, 4 and 7, approximately 8 h prior to

sample collection. Gas collection and flux calculations followed the methods described in the previous section.

4.2.3 Three-day N_2O campaigns

Variability in N_2O flux measurements is generally high and can be exacerbated by greater soil N availability (Clayton 1994). As such, complex treatment interactions affecting N_2O fluxes can be difficult to assess during these periods (Choudhary et al. 2002). To measure the impact of [CO_2], water supply and their interactions on N_2O fluxes, I measured emissions from all [CO_2] and irrigation treatment combinations following the resolution of N_2O peaks, i.e. in the few weeks following urea application. Gas samples from all sectors were collected over three consecutive days on 5-7 September 2016, 29-31 October 2016, 14-16 February 2017 and 1-3 March 2017. I focused the sampling efforts on spring and autumn when production and subsequent plant responses to eCO_2 would be highest. Samples were collected 10 d after urea application in October, 9 d after in March, 28 d after in September and 34 d after in February. Plots were irrigated approximately 8 h prior to the first sample collection. Sample collection was performed per the static chamber method described previously. Daily measurement of all 60 sectors necessitated collecting samples in 4 complete blocks of 15 sectors. Immediately upon completion of one block, the technician would begin sampling the subsequent block. This resulted in approximately 80 mins between the start of one block and next block.

4.2.4 Statistical analysis

I used linear mixed effects models with repeated measures to assess the impact of [CO_2] and irrigation treatments on N_2O emissions using the LME4 package (Bates et al. 2015) in R version 3.5.0 (R Core Team 2018). In the 7-d campaign, I assessed the impact of experimental treatments on N_2O emissions using a full model containing CO_2, date and CO_2 × date interaction as fixed effects with sector number as a random effect. I performed model selection using the Likelihood Ratio Test, in which I used ANOVA to compare the full model to the full model with a single term dropped to obtain P-values for all model terms (Vuong 1989). The final model obtained through this process contained CO_2 and date as fixed factors with sector number as a random effect (Table 4.1). I performed pairwise comparisons for CO_2 using the R package 'emmeans' (Lenth 2018). Box-Cox and diagnostic plots were used to check data for heteroscedasticity and normality. Data were transformed where necessary to avoid violation of model assumptions.

N_2O emissions and treatment impacts on soil characteristics in the 3-d campaigns were similarly analysed using linear mixed effects models. Within campaign N_2O emissions were consistent between days, thus mean emissions were used for all analyses. To assess treatment impacts on N_2O emissions, I constructed a full model for each 3-d campaign containing CO_2,

Table 4.1. Model selection results for the 7-d campaign in October 2017. P-values were calculated using the Likelihood Ratio Test. Bold text indicates P value ≤ 0.05. Underlined text indicates 0.05 < P-value < 0.1.

Fixed effects	df	Chi sq.	P-value
CO_2 × date	12	8.6	0.7
CO_2	1	5.1	<u>0.07</u>
date	6	83.5	< **0.001**
Random effects			
Std. deviation: sector = 0.54; residuals = 0.18			

irrigation and CO_2 × irrigation as fixed factors as well as WFPS, NH_4^+ and NO_3^- as covariates and block as a random effect. I performed model selection using the Likelihood Ratio Test, which provided P-values for construction of the final model for each campaign (Table 4.2). For each campaign, the final model contained CO_2 as the only fixed effect with block as a random effect. Covariates were included only for $P \leq 0.05$ (Table 4.3). I then performed pairwise comparisons for CO_2 using 'emmeans'. As in our methods for the 7-d campaign, diagnostic plots were used to check data for normality and were transformed when necessary to avoid violation of model assumptions. By this same method, I constructed simple linear mixed effects models with pairwise comparison to identify significant differences in soil WFPS, NH_4^+, NO_3^- and plant N between CO_2 levels.

4.3 Results

N_2O emissions in the TasFACE2 experiment varied substantially over time and were highly dependent upon the time since nitrogenous fertiliser was applied. Peak emissions were approximately 9,200 µg N_2O-N m^{-2} h^{-1} on day 1 but declined rapidly to 2,100 µg N_2O-N m^{-2} h^{-1} on day 7 (Fig. 4.1). Mean fluxes measured ~10 d following urea application were 260 µg N_2O-N m^{-2} h^{-1} in spring and 190 µg N_2O-N m^{-2} h^{-1} in autumn (Fig. 4.2). Four or so weeks after urea application, N_2O flux had declined to only 20 µg N_2O-N m^{-2} h^{-1} in spring and 13 µg N_2O-N m^{-2} h^{-1} in autumn (Fig. 4.2). These variations in N_2O flux corresponded with soil NO_3^- availability, which was about 4 times higher in both spring and autumn approximately 10 d following urea application than 30 d after application (Fig. 4.3).

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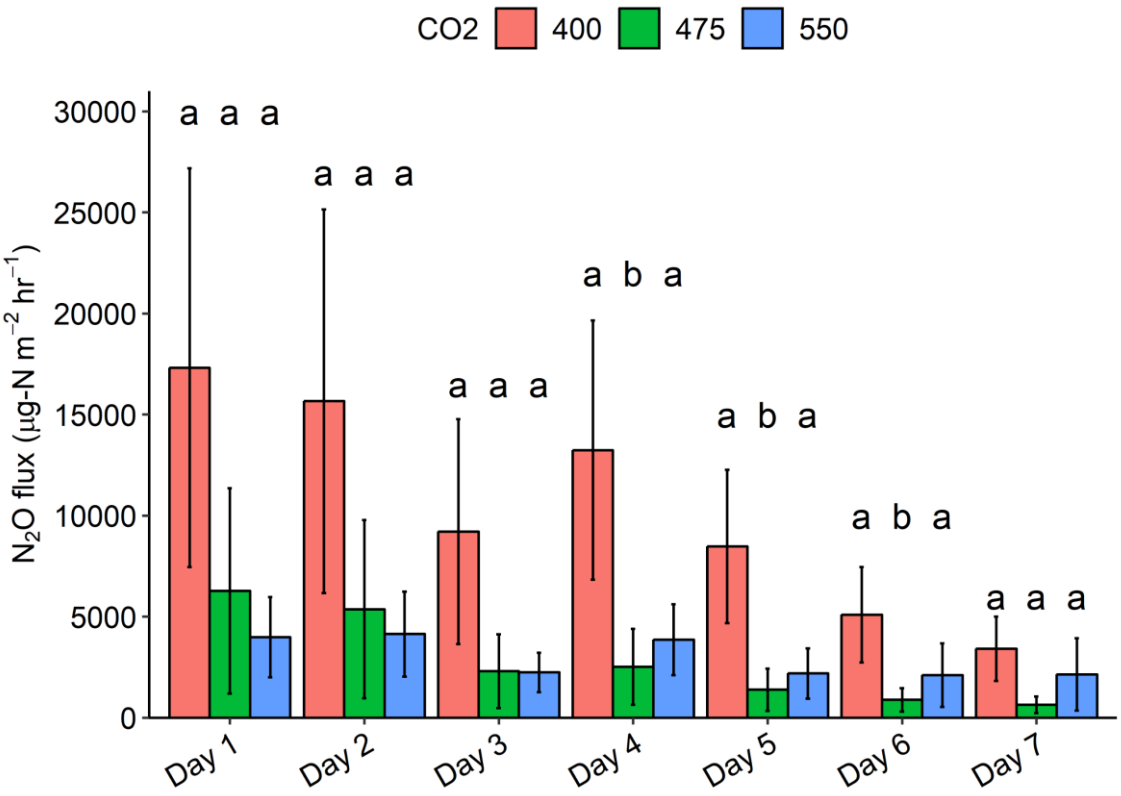

Figure 4.1. N_2O emissions from soils under three levels of CO_2 (400, 475 and 550 µmol CO_2 mol^{-1}) following urea application. Presented are daily means ± SEM measured over 7 days in October 2017. All sectors were watered at the same time, approximately 8 h prior to gas sampling on day 1 and day 4. Those means with the same letter within a day are not significantly different ($P > 0.05$).

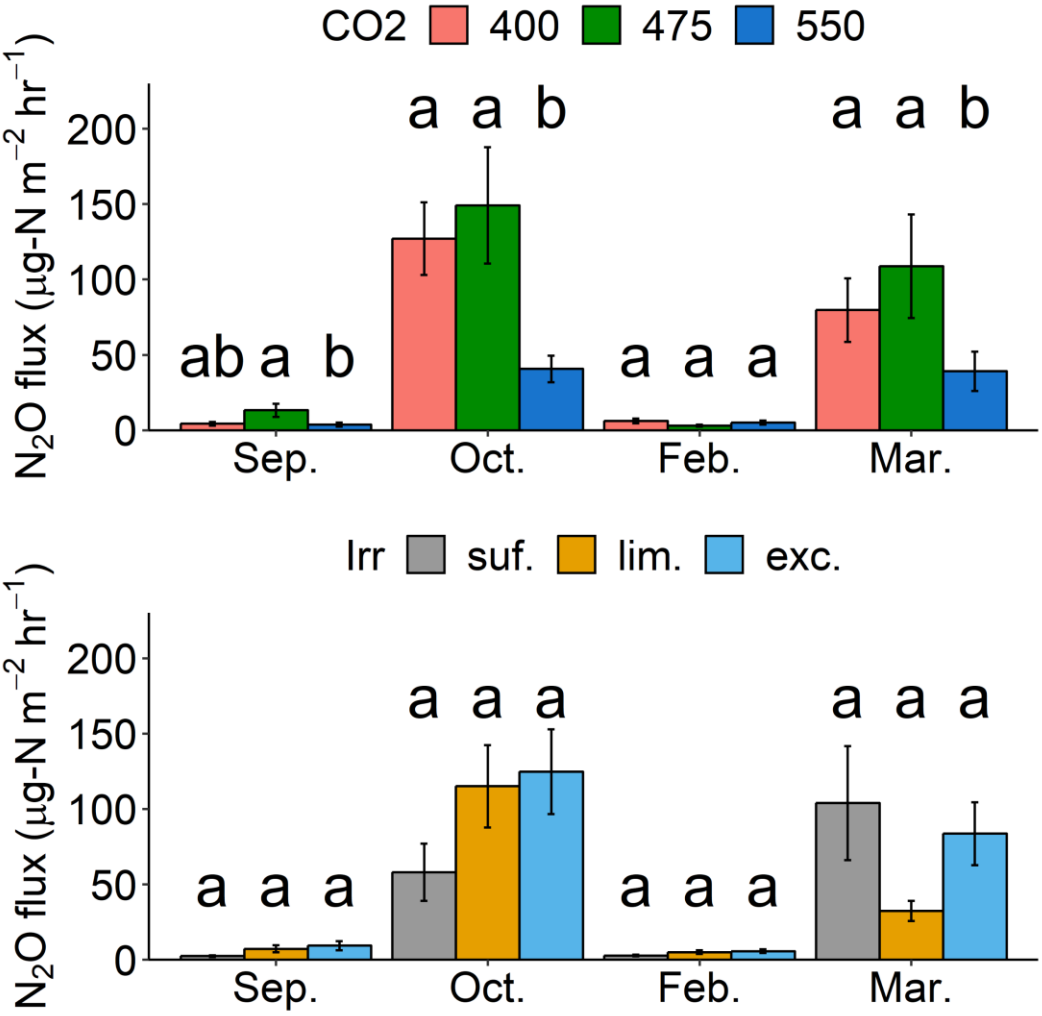

Figure 4.2. The impact of [CO_2] (top) and irrigation treatment (bottom) on N_2O emissions from soils following application of urea. Sectors were exposed to one of three CO_2 levels (400, 475 and 550 µmol CO_2 mol^{-1}) and one of three irrigation treatments (*sufficient*, *limit* and *excess*). Reported are mean fluxes ± SEM averaged over 3-d sampling campaigns ~30 d after application of urea in September 2016 and February 2017 and ~10 d after application of urea in October 2016 and March 2017. Those means with the same letter within a month are not significantly different ($P > 0.05$).

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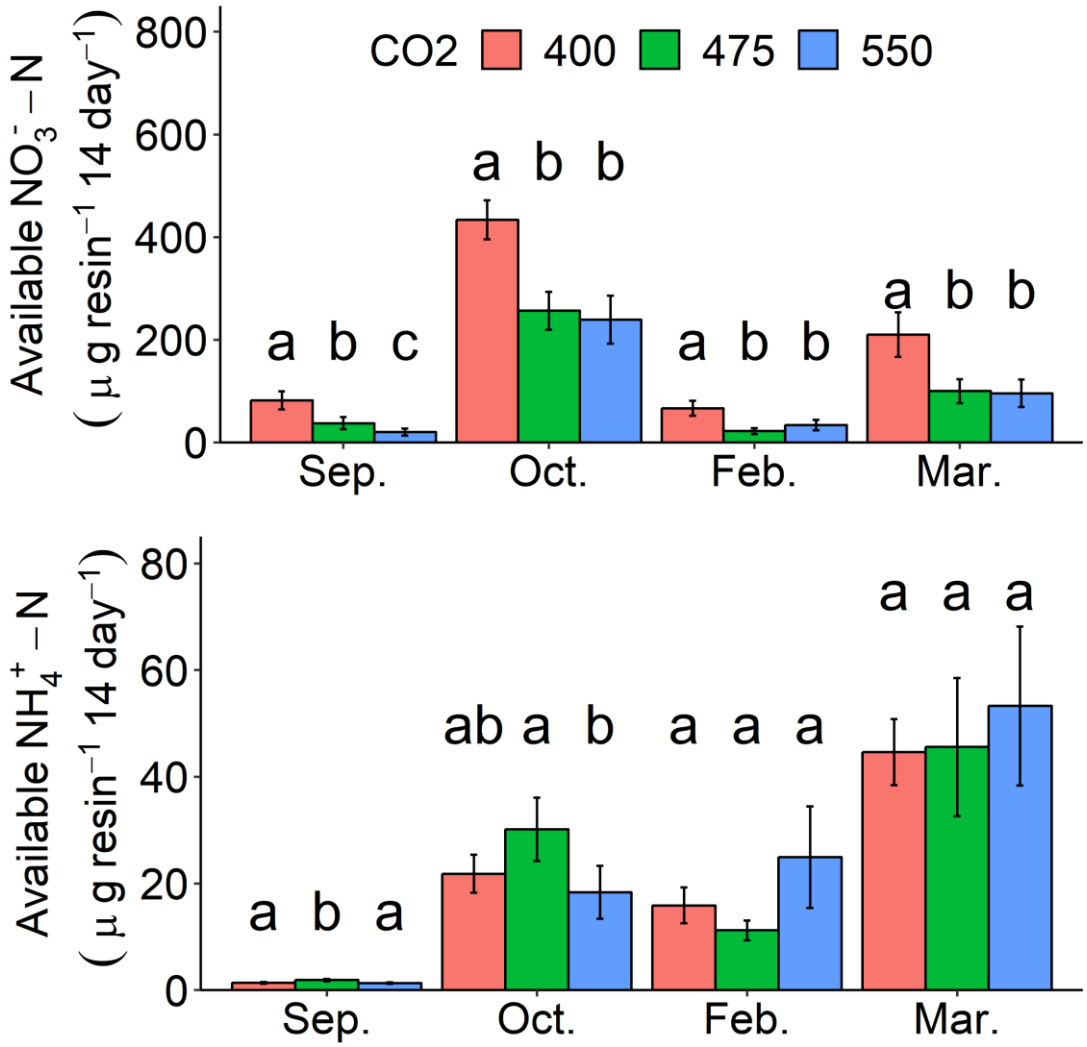

Figure 4.3. Seasonal variability of mean ion-exchange membrane available nitrate (NO_3^-; top) and ammonium (NH_4+; bottom) measured in soils under one of three CO2 levels (400, 475 and 550 µmol CO_2 mol^{-1}). Membranes were deployed on 14-d intervals in spring 2016 (September and October) and autumn 2017 (February and March). Those means with the same letter within a month are not significantly different ($P > 0.05$).

The effect of [CO_2] on N_2O flux varied between campaigns and CO_2 levels, but there was a strong tendency for eCO_2 to reduce emissions (Figs. 4.1 and 4.2). In the 7-d campaign in spring, mean N_2O flux was 70% lower under 475 µmol CO_2 mol^{-1} compared to 400 µmol CO_2 mol^{-1}. Although 550 µmol CO_2 mol^{-1} had a similar effect on mean N_2O emissions over this period, fluxes from 550 µmol CO_2 mol^{-1} were not significantly different from 400 µmol CO_2 mol^{-1} (Fig. 4.1). Mean N_2O flux under 400 µmol CO_2 mol^{-1} was 10,300 µg N_2O-N m^{-2} h^{-1} while mean fluxes under 475 and 550 µmol CO_2 mol^{-1} were 2,800 and 3,000 µg N_2O-N m^{-2} h^{-1} respectively. Interestingly, N_2O emissions approximately 10 d following urea application in spring and autumn were also reduced under 550 µmol CO_2 mol^{-1} by 60% relative to 400 µmol CO_2 mol^{-1}, but no similar effect was present under 475 µmol CO_2 mol^{-1} over the same periods (Fig. 4.2 and Table 4.2). However, N_2O emissions were similar between CO_2 levels approximately 30 d after urea application in spring and autumn (Fig. 4.2 and Table 4.2). These results demonstrate that the CO_2-effect on N_2O emissions was measurable only in periods of moderate to high soil mineral N availability.

In October 2017, WFPS was approximately 60% and remained consistent across the 7-d period. Surprisingly, all sectors had similar WFPS throughout this campaign regardless of CO_2 level (Table 4.4). In the 7-d campaign, I measured N_2O emissions in *sufficient* sectors only, thus irrigation had no impact here. There was a greater range of WFPS in the 3-d campaigns, with values ranging from 20.9% WFPS to 95.4% WFPS (Table 4.5). WFPS was higher in sectors exposed to eCO_2 compared to those under ambient CO_2, but no differences were present between 475 and 550 µmol CO_2 mol^{-1} (Table 4.5). The strength of the CO_2-effect on WFPS varied between months, with an increase of 27% in September, 6% in October 2016, 17% in February and 11% in March (Table 4.5). There was a significant impact of irrigation on WFPS by month (χ (6) = 23.0, P <0.001), which amounted to lower

879 WFPS in *limit* plots relative to *sufficient* and *excess* ($P < 0.001$). The suppressive effect of
880 CO_2 on N_2O emissions was present in numerous campaigns regardless of WFPS (Figs. 4.1
881 and 4.2). Importantly, there was neither a significant impact of WFPS on N_2O emissions
882 (Table 4.3) or was there a CO_2 × irrigation interaction (χ (4) = 0.9, $P > 0.5$). This
883 demonstrates the CO_2-effect on N_2O emissions was consistent across irrigation treatments
884 and WFPS.

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Table 4.2. Estimated effect of [CO_2] and irrigation on N_2O emissions (µg N_2O-N m^{-2} h^{-1}). Coefficients and SE were estimated using a linear mixed effects model which contained [CO_2] (400, 475 and 550 µmol CO_2 mol^{-1}) and irrigation (*sufficient*, *limit* and *excess*) as fixed factors and block as a random effect. The lower and upper bounds of the 95% confidence interval are reported. P-values were calculated with the Likelihood-Ratio Test. Bold text indicates P value ≤ 0.05. Underlined text indicates 0.05 ≤ P-value < 0.1.

Month	Variable	Coef.	SE	L. CI.	U. CI.	Chi sq.	df	P-value
Sep. '16	Intercept	2.6	1.3	1.5	4.4	-	-	-
	CO2 [475]	2.1	1.3	1.2	3.5	10.0	2	**0.007**
	CO2 [550]	0.9	1.3	0.5	1.5			
	Irr. *Limit*	1.9	1.4	1.0	3.5	4.9	2	<u>0.09</u>
	Irr. *Excess*	1.7	1.3	1.0	3.0			
	Random effects standard deviation: block = 0; residuals = 0.3							
Oct. '16	Intercept	53.7	1.5	23.4	123.3	-	-	-
	CO2 [475]	0.8	1.5	0.4	1.9	11.2	2	**0.003**
	CO2 [550]	0.3	1.5	0.1	0.6			
	Irr. *Limit*	2.2	1.6	0.9	5.6	3.5	2	0.2
	Irr. *Excess*	1.9	1.5	0.9	4.3			
	Random effects standard deviation: block = 0.1; residuals = 0.5							
Feb. '17	Intercept	4.1	1.3	2.6	6.4	-	-	-
	CO2 [475]	0.7	1.3	0.4	1.0	3.5	2	0.2
	CO2 [550]	0.9	1.3	0.6	1.4			
	Irr. *Limit*	1.4	1.3	0.8	2.3	2.7	2	0.3
	Irr. *Excess*	1.5	1.3	0.9	2.3			
	Random effects standard deviation: block = 0.02; residuals = 0.3							
Mar. '17	Intercept	58.0	1.6	21.4	157.2	-	-	-
	CO2 [475]	0.9	1.5	0.4	1.8	7.8	2	**0.02**
	CO2 [550]	0.4	1.5	0.2	0.8			
	Irr. *Limit*	0.6	1.5	0.2	1.4	2.5	2	0.3
	Irr. *Excess*	1.1	1.5	0.5	2.3			
	Random effects standard deviation: block = 0.2; residuals = 0.5							

Table 4.3. Impact of soil characteristics on N_2O emissions in TasFACE2 between September 2016 and March 2017. Soil parameters tested in the linear mixed effects model include water-filled pore space (WFPS), ammonium (NH_4^+) and nitrate (NO_3^-). P-values were calculated using the Likelihood Ratio Test. Bold text indicates P value ≤ 0.05. Underlined text indicates 0.05 ≤ P-value < 0.1.

Month	Variable	df	Chi sq.	P-value
Sep. '16	WFPS	1	0.1	0.7
	NH_4^+	1	5.5	**0.02**
	NO_3^-	1	9.7	**0.002**
Oct. '16	WFPS	1	0.6	0.4
	NH_4^+	1	0.001	0.9
	NO_3^-	1	6.4	**0.01**
Feb. '17	WFPS	1	0.02	0.9
	NH_4^+	1	0.09	0.8
	NO_3^-	1	0.4	0.5
Mar. '17	WFPS	1	2.8	<u>0.09</u>
	NH_4^+	1	1.1	0.3
	NO_3^-	1	2.8	<u>0.09</u>

The variation in N_2O fluxes between sampling dates demonstrates the strong impact of urea application and mineral N availability on N_2O fluxes (Fig. 4.1, Fig. 4.2). Within-campaign N_2O fluxes varied as a function of NO_3^- (Table 4.3), with a significant positive association in September (r^2= 0.08) and October (r^2 = 0.20). Thus, soil NO_3^- explains a small proportion of overall emissions. Soil NO_3^- availability in the 3-d campaigns was lower under eCO_2 by between 40% and 75%, but no differences in NO_3^- were present between 475 and 550 µmol CO_2 mol^{-1} in any period (Fig. 4.3). In contrast, NH_4^+ remained similar between CO_2 levels except for a minor increase in 475 µmol CO_2 mol^{-1} in September and October 2016 (Fig. 4.3) and had little impact on N_2O emissions (Table 4.3) possibly due to substantially lower concentrations of NH_4^+ compared to NO_3^- (Fig. 4.3). Soil mineral N availability was indirectly affected by eCO_2 through increased plant production with [CO_2]. The total N incorporated into new root biomass increased with [CO_2] (P < 0.001), resulting in more N incorporated into roots under 550 µmol CO_2 mol^{-1} than in soils exposed to 400 µmol CO_2 mol^{-1} (P < 0.001) or 475 µmol CO_2 mol^{-1} (P < 0.03). The total N contained in aboveground

Table 4.4. Impact of CO_2 treatment on soil water content and soil temperature in plots under three levels of CO_2 (400, 475 and 550 µmol CO_2 mol^{-1}) with *sufficient* irrigation. Reported is mean percent water-filled pore space (WFPS) ± SEM from 0 - 5 cm and mean temperature ± SEM at 20 cm measured throughout a 7-d campaign which commenced 17 October 2017.

Variable	Day	400 µmol CO_2 mol^{-1}		475 µmol CO_2 mol^{-1}		550 µmol CO_2 mol^{-1}	
%WFPS	1	53.7	± 10.9	44.7	± 7.4	64.0	± 9.1
	4	58.3	± 9.9	54.0	± 8.3	66.3	± 6.7
	7	61.2	± 10.6	57.1	± 1.3	65.1	± 6.4
Temp. (ºC)	1	23.3	± 1.3	23.7	± 1.4	23.5	± 1.2
	4	23.4	± 0.8	23.0	± 0.6	23.0	± 0.3
	7	21.4	± 0.2	21.3	± 0.1	21.2	± 0.1

biomass was lower under eCO_2 ($P < 0.03$; Fig. 4.4). Total N contained in shoots grown under 400 µmol CO_2 mol^{-1} was higher than 550 µmol CO_2 mol^{-1} ($P < 0.03$) but was similar to 475 µmol CO_2 mol^{-1} ($P = 0.09$). The CO_2-effect on total N stored in plants was driven by increased plant biomass, specifically roots, under eCO_2 rather than changes in plant tissue quality. The stimulation of root production with [CO_2] increased N storage in belowground biomass while a modest decrease in shoot production with [CO_2] lowered N contained in aboveground biomass (Figs. 4.4, S1). The difference in total root N between CO_2 levels was present throughout the experiment with the greatest contrast in spring (Fig. 4.4). During this season, aboveground production remained similar between treatments, suggesting plant N demand increased during this period (Fig. 4.4).

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Table 4.5. Mean percent water-filled pore space (WFPS) ± SEM from 0 - 5 cm in plots under three levels of CO_2 (400, 475 and 550 µmol CO_2 mol^{-1}) and three irrigation volumes (*sufficient*, *limit*, and *excess*). Data were averaged over gas sampling campaigns in September 2016, October 2016, February 2017 and March 2017.

WFPS							
Month	Irrigation	400 µmol CO_2 mol^{-1}		475 µmol CO_2 mol^{-1}		550 µmol CO_2 mol^{-1}	
Sep. ‘16	*Limit*	53.2	± 4.3	77.1	± 2.9	74.4	± 7.0
	Sufficient	60.2	± 3.2	74.2	± 4.7	79.3	± 6.7
	Excess	58.2	± 5.6	66.3	± 3.8	71.4	± 4.4
Oct. ‘16	*Limit*	74.2	± 4.4	72.8	± 8.1	81.1	± 8.0
	Sufficient	83.9	± 4.6	86.3	± 4.0	95.4	± 1.8
	Excess	85.3	± 2.3	83.5	± 2.5	88.7	± 2.8
Feb. ‘17	*Limit*	20.9	± 3.5	27.6	± 7.4	26.8	± 4.7
	Sufficient	30.7	± 1.4	30.0	± 3.7	40.3	± 9.2
	Excess	32.9	± 2.1	35.9	± 4.7	36.5	± 4.0
Mar. ‘17	*Limit*	26.1	± 4.7	33.0	± 3.1	34.7	± 5.1
	Sufficient	46.4	± 2.7	44.9	± 3.1	45.0	± 4.1
	Excess	41.5	± 1.0	50.4	± 2.8	44.5	± 2.0

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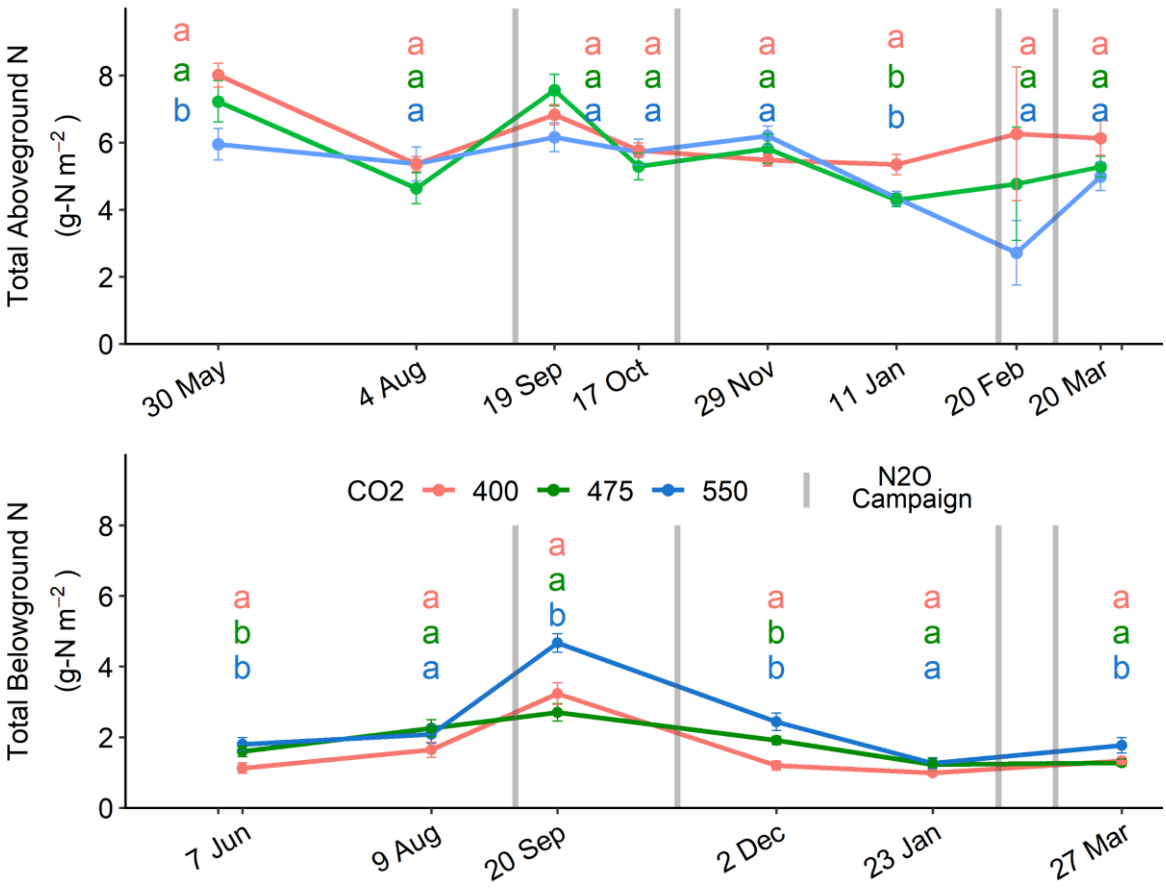

Fig. 4.4. Mean total nitrogen ± SEM contained in plant biomass during N_2O flux measurement campaigns from May 2016 through March 2017. N contained in aboveground plant biomass (top) was measured through periodic harvest of all biomass. Belowground plant N (bottom) was estimated using root cores. Mean N was determined using a CHN elemental analyser. Those means with the same letter within a date are not significantly different ($P > 0.05$).

4.4 Discussion

It was unclear whether N_2O emissions would be higher under eCO_2 than in control plots due to plant-driven increases in belowground organic matter input and soil water content or if plant-mediated declines in mineral N would drive changes in N_2O emissions. These are the primary factors underlying denitrification rates and consequently N_2O production in upland soils (Burford and Bremner 1975, van Groenigen et al. 2011, Saggar et al. 2013). Surprisingly, N_2O fluxes in TasFACE2 were never higher under eCO_2 and were often substantially lower than emissions under ambient CO_2 despite an increase in belowground C allocation and soil moisture. Our findings indicate that lower N_2O emissions were associated with a decline in soil NO_3^- under eCO_2 and were independent of soil water content. To determine the persistence of the CO_2-effect on N_2O emissions after urea application, I measured emissions for 7 days immediately after fertiliser application, as well as over four 3-d campaigns approximately 10 and 30 days after urea application. Results from the 7-d campaign confirmed that the majority of emissions occurred during this period as has been observed broadly (Clayton 1994, Baggs et al. 2003b, Kettunen et al. 2007, Kammann et al. 2008), while the 3-d campaigns had much lower emissions compared to the peak. Importantly, these findings show that the suppressive effect of eCO_2 on soil N_2O emissions was present during both peak and baseline emission periods and occurred regardless of the prevailing soil water content.

Nitrification may have been a considerable source of N_2O in this experiment for all CO_2 levels. Net nitrification can be increased under eCO_2 (Carnol et al. 2002, Barnard et al. 2005, Nguyen et al. 2019) (Zak et al. 1993, Cheng et al. 2001, Ebersberger et al. 2003, McKinley et al. 2009, Björsne et al. 2014) and is further stimulated through the application of nitrogenous fertiliser (Stanford and Smith 1972, Barnard et al. 2005). Although nitrification releases less

N_2O than denitrification (Mathieu et al. 2006), high NH_4^+ oxidation rates can have a strong impact on emissions following application of ammonium or ammonium yielding fertilisers such as urea (Tortoso and Hutchinson 1990, Bremner 1997, Zhu et al. 2013b). In TasFACE2, the low IEM-available levels of NH_4^+ compared to NO_3^- in a system reliant on N from urea is indicative of high nitrification rates (Fig. 4.3). NH_4^+ levels were a fraction of NO_3^- levels, demonstrating that nearly all urea applied was ammonified then nitrified to NO_3^-. Furthermore, this trend was present in all seasons regardless of WFPS (Tables 4.4 and 4.5), suggesting nitrification and associated emissions were not inhibited by increased WFPS. Immobilisation and oxidation undoubtedly decreased free soil NH_4^+ rapidly, limiting the impact of nitrification to the days immediately following urea application when free NH_4^+ levels were high.

Denitrification was probably a major source of N_2O in this system but was likely to have been constrained by NO_3^- availability. Heterotrophic denitrification requires anaerobic conditions for the reduction of NO_3^- and typically occurs only in soils above 60% WFPS (Firestone and Davidson 1989a, Arnone and Bohlen 1998, Kammann et al. 2008), indicating that this pathway only could have been a major process in spring when soil water content exceeded this threshold (Bollmann and Conrad 1998, Bateman and Baggs 2005). However, lower emissions under eCO_2 were observed throughout the experiment regardless of soil water content, indicating that heterotrophic denitrification cannot explain this pattern alone. Nitrifier denitrification could have been a source of N_2O in this system. Similar to heterotrophic denitrification, N_2O is released as an intermediary during NO_3^- reduction, but nitrifier denitrification is paradoxically carried out by nitrifiers rather than heterotrophs (Wrage et al. 2001). N_2O produced via nitrifiers can comprise up to 80% of total emissions depending on soil conditions (Webster and Hopkins 1996, Gödde and Conrad 1999).

Importantly, this pathway is insensitive to soil aerobic status relative to heterotrophic denitrification but similarly requires NO_3^- (Wrage et al. 2001, Kool et al. 2011, Zhu et al. 2013a). Therefore, it is likely this was a source of N_2O in this system given our analysis indicated N_2O emissions were unaffected by WFPS but were impacted by NO_3^- availability (Table 4.3). Additionally, heterotrophic denitrifiers and associated N_2O emissions are commonly stimulated by organic C input under eCO_2 (Baggs et al. 2003a). Therefore, I would have expected to see some stimulation in N_2O emissions due to the CO_2-effect on root activity here as this is a major energy source for heterotrophs in grasslands (Pendall et al. 2004, Iversen et al. 2008). This further supports our conclusion that most of the emissions were driven by nitrifiers rather than heterotrophs, as nitrifiers are largely unaffected by organic matter inputs (Shaw et al. 2006). The contribution of each of these pathways could have been quantified through the use of isotopic methods (Kool et al. 2010). However, given the role of NO_3^- and water content on N_2O emissions here as well as the persistence of the CO_2-effect on emissions over time provides evidence for the importance of nitrifier denitrification in this grassland.

Most studies report increased N_2O emissions under eCO_2 due primarily to stimulation of denitrification through plant-mediated reductions in soil aerobic status (Arnone and Bohlen 1998, Baggs et al. 2003a, Kammann et al. 2008, van Groenigen et al. 2011). However, several studies have demonstrated that N availability, rather than soil water content, constrains denitrification rates (Ambus and Robertson 1999, Mosier et al. 2002). Plant growth is often stimulated by exposure to eCO_2, increasing plant demand for N, potentially reducing soil N availability (Hungate et al. 1997a, Pleijel et al. 1998, Baggs et al. 2003b, Kettunen et al. 2007). In a grassland mesocosm, Kettunen et al. (2007) found the strongest suppression of emissions by eCO_2 occurred during germination, when seedling N

requirements were high. A similar effect was present in the eighth year of CO_2 fumigation of a managed grassland, in which N_2O emissions were reduced under eCO_2 immediately following aboveground biomass harvest and N application (Baggs et al. 2003b). Similarly, in the first growing season in TasFACE2, the suppressive effect of CO_2 on emissions was strongest in spring (Fig. 4.2) when production and plant N demand were highest (Fig. 4.4). Total plant N in this system was driven by root production (Fig. S1), which increased N demand despite a decline in total aboveground N (Fig. 4.4). Furthermore, increased root biomass results in greater surface area for nutrient uptake (Finzi et al. 2007). This enhancement of plant N acquisition and immobilisation under eCO_2 could limit microbial access to N, resulting in reduced N_2O emissions.

Under eCO_2, declines in N availability, particularly NO_3^-, may be driven by CO_2-stimulated plant production, which increases the total N immobilized in plant biomass (Luo et al. 2004, de Graaff et al. 2006, Hovenden et al. 2008, Dijkstra et al. 2010a). However, Hovenden et al. (2008) reported declines in N availability without a corresponding increase in plant production. If plant production was the driver of declining mineral N, then this effect only would have occurred when the plant biomass response was present. Thus, plant immobilization of N may be a contributing factor but cannot explain the decline of soil NO_3^- alone. Furthermore, Hovenden et al. (2008) found the depletion of NO_3^- was prevented through simultaneous application of warming. Warming has the tendency to dry soils, which could lower the CO_2-effect on soil water content, thereby suppressing denitrification rates and associated N losses. Therefore, it is worth considering that eCO_2 stimulation of denitrification in TasFACE2 may have lowered NO_3^- availability despite no increase in N_2O emissions under eCO_2. Through denitrification, gaseous N losses can occur as NO, N_2O and N_2, the ratio of these products being partly determined by soil water content (Weier et al.

1993). The N_2:N_2O end-product ratio increases at high water content (Weier et al. 1993), suggesting the CO_2-effect on WFPS in this study may have depleted soils of NO_3^- through complete reduction to N_2 via denitrification. Thus, N_2O emissions may have been lower under eCO_2 without a corresponding reduction in denitrification. While this is theoretically possible in TasFACE2, I am unable to prove this without additional measurements. Isotopic methods provide the most reliable assessment of N_2 and N_2O emissions from soils (Stevens and Laughlin 1998), though denitrification enzyme activity could have elucidated whether there was a stimulation of denitrification despite lowered N_2O emissions (Brooks et al. 1992). These measurements are beyond the scope of this study but warrant future investigation.

I expected N_2O fluxes to follow a linear relationship with [CO_2] due to the linear increase in belowground C input (Gill et al. 2002a) and soil water content (Ainsworth and Rogers 2007) in other studies. Instead, emissions decreased under eCO_2 treatment in some cases but remained similar to ambient CO_2 in others. This is unsurprising given many of the soil properties underlying N-transformation pathways, such as WFPS, soil NO_3^- and C input, were found to have non-linear responses to [CO_2] in this system. However, it is less obvious why the response of these characteristics did not follow a linear trend with [CO_2]. I expected WFPS would increase with [CO_2] due to a linear response in stomatal conductance and water-use efficiency found elsewhere (Polley et al. 1993). Although eCO_2 substantially increased WFPS at TasFACE2, the lack of difference between 475 and 550 µmol CO_2 mol^{-1} could be due to the challenges associated with detection of modest difference in field water content. Similarly, I also expected a linear increase in primary production with [CO_2] (Gill et al. 2002a), but instead found a linear increase in sectors exposed to *sufficient* irrigation only and similar production rates between 475 and 550 µmol CO_2 mol^{-1} in *excess* and *limit* irrigation treatments (data not shown). NO_3^- was lower under eCO_2 but similar between 475

and 550 µmol CO_2 mol^{-1} as well. Given that N transformations and subsequent gaseous N losses are regulated by WFPS, I would expect that the CO_2-effect on both WFPS and NO_3^- would follow a similar trend. The expectation that changes in C, N and soil water availability due to plant responses to [CO_2] affect N_2O emissions in a linear trend risks oversimplifying the complex factors that regulate N transformations.

In conclusion, I demonstrate that the suppressive effect of CO_2 on N_2O emissions was driven largely by declines in soil NO_3^- rather than increased soil water content. In fact, the suppression of N_2O emissions persisted across seasons and a range of WFPS, demonstrating that this effect is generalisable in this system. Under eCO_2, declines in free NO_3^- were due partly to increased production of roots and greater plant N demand, though increased N immobilisation may not fully explain the decline in NO_3^-. Increased WFPS under eCO_2 may have stimulated the depletion of soil NO_3^- via denitrification. This may have been associated with a higher denitrification N_2:N_2O end-product ratio, which increased soil N_2 emissions rather than N_2O emissions. The testing of this hypothesis through measurement of N_2 and N_2O is beyond the scope of this study and warrants future investigation. Our findings differ from many studies which report plant responses to eCO_2 stimulate N_2O emissions rather than a suppress as I observed here. This demonstrates that plant responses to CO_2 can have varying impacts on N_2O emissions from grasslands with divergent outcomes for climate change.

5 EFFECT OF ELEVATED CARBON DIOXIDE ON SOIL RESPIRATION AND DECOMPOSITION

5.1 Introduction

Soils are one of the largest natural sources of CO_2 to the atmosphere and annually release approximately 11 times more CO_2 than is produced through anthropogenic sources (Raich et al. 2002). Soil respiration (R_S) is the sum of heterotrophic respiration and autotrophic respiration, thus is determined by the activity of plants and soil microbes (Singh and Gupta 1977). R_S is impacted by global changes such as rising atmospheric CO_2 and changing precipitation patterns because plant and soil microbial processes are sensitive to soil moisture and carbon availability (Castro et al. 2010, Dieleman et al. 2012). Given atmospheric [CO_2] has risen from 280 ppm to over 400 ppm and is projected to continue rising (IPCC 2014), it is likely plant responses to changing [CO_2] will impact R_S by altering the processes underlying this flux. Due to the magnitude of R_S on a global level, even minor shifts in this flux may have major implications for climate change (IPCC 2014).

Plants, and roots in particular, play a key role in R_S because roots are a direct source of CO_2 to the atmosphere and are a major source of organic matter for decomposition (Zak et al. 2000, Iversen et al. 2008, Solly et al. 2014). Increased photosynthate supply in grasses under elevated CO_2 (eCO_2) stimulates autotrophic respiration directly through greater allocation of plant C to root production and maintenance (de Graaff et al. 2006, Lee et al. 2010). As a greater proportion of total photosynthate is allocated belowground to roots under eCO_2, heterotroph activity may be stimulated through increased substrate supply available to microbes (van Groenigen et al. 2017). This effect has been well-studied in experiments comparing R_S in grasslands under ambient CO_2 to a single eCO_2 level (Adair et al. 2011, Selsted et al. 2012, Osanai et al. 2015, Ryan et al. 2015). However, relatively little is known about the response of plants and R_S to further increases in [CO_2]. Gill et al. (2002b) found

that photosynthesis increases with [CO_2] in some C_3 plants across a [CO_2] gradient. This could stimulate R_S through greater root production and consequently autotrophic respiration but also by increasing heterotroph activity through provision of additional C belowground. The response of R_S and litter decomposition to two [CO_2] levels has been well-studied but it is unclear if these processes will increase with [CO_2] or if complex factors may limit this effect.

Water supply is a key factor affecting R_S due to the positive influence of soil moisture on plant production and microbial activity (Raich and Schlesinger 1992). Models predict positive radiative forcing from increasing greenhouse gas concentrations will result in drying of soils in low- and mid-latitude regions (Seager et al. 2007, Dai 2011, Trenberth et al. 2013). Decreased rainfall frequency due to climate change may lead to longer periods without rainfall and greater occurrence of drought (Allan and Soden 2008, Singh et al. 2013). [CO_2] will indirectly affect R_S by affecting plant-water relations which can impact soil water availability. Plant stomatal conductance is decreased under eCO_2, resulting in lower evapotranspiration and greater plant water-use efficiency (Nelson et al. 2004, Martins et al. 2016). This effect increases soil moisture (Leakey et al. 2009) and may stimulate biological activity through alleviation of osmotic stress under water limited conditions and increase substrate diffusion to microbes (Morgan et al. 2011). R_S may increase under such conditions, with evidence from previous studies demonstrating that when present, the CO_2 effect on R_S increases in dry soils (Pendall et al. 2003, Morgan et al. 2011, Lu et al. 2018). Thus, the negative impacts of water stress on R_S due to climate change may be mitigated by increased plant water-use efficiency under eCO_2. However, it remains unclear to what extent plant responses to [CO_2] may affect soil moisture. Plant water-use efficiency in C_3 grasses have been shown to increase linearly with [CO_2] through 550 μmol CO_2 mol^{-1} (Polley et al. 1993, Anderson et al. 2001b). This suggests additional supply of moisture with [CO_2] could

increase the R_S response through further alleviation of water stress in dry soils. Such a generalisation could help improve our predictions of R_S from grasslands under future climate conditions.

Here I analyse the independent and combined effects of [CO_2] and soil water supply on C cycling in a temperate grassland using the TasFACE2 experiment. Specifically, the goals of this study were to assess the impact of three levels of [CO_2] (400, 475 and 550 μmol CO_2 mol^{-1}) and three levels of water supply (*sufficient*, *limit* and *excess*) on R_S and root decomposition. Experimental [CO_2] treatments were delivered using free-air CO_2 enrichment (FACE) technology and water supply was controlled through the exclusion of all precipitation coupled with irrigation. As TasFACE2 allows the independent control of [CO_2] and water supply, the effect of both belowground C input and soil water content can be tested. I hypothesized that [CO_2] would stimulate R_S and root decomposition through plant-mediated impacts on C availability rather than through impacts on soil water content. Given both belowground C and soil water content likely increase with [CO_2], I expected the CO_2 effect size to increase with [CO_2] regardless of the process driving the CO_2-effect on C cycling here. Increases in soil moisture with [CO_2] were predicted to be strong drivers of R_S, an effect which would be largest in soils with low water content.

5.2 Methods

5.2.1 Soil CO_2 flux

R_S was measured for all sectors in four campaigns between May 2016 and January 2017. I inserted one polyvinyl chloride collar (diameter 10 cm, height 10 cm) 8 cm into the soil in each sector. As collar installation has been shown to reduce autotrophic respiration through severing of roots (Heinemeyer et al. 2011), I mitigated this effect by allowing a 30-d

Figure 5.1. Photo of soil respiration (R_S) measurements collected using a Li-6400 infrared gas analyser with Li-6400-09 soil efflux chamber in TasFACE2.

regrowth period prior to R_S measurements. Vegetation was unaltered for measurements. Efflux measurements were made in each CO_2 treatment block (i.e. one plot at 400, 475 and 550 μmol mol^{-1}; Fig. 2.2) for three consecutive days beginning with the day of watering. Three to five measurements were collected from each sector between 9:00 and 15:00 hours, thus at least six hours after the night-time irrigation on the day of watering. All four blocks were sampled by this method within a 14-d period for each campaign. Campaigns were from 23 May – 4 June 2016, 13 September – 28 September 2016, 21 November – 2 December 2016 and 2 January – 18 January 2017. R_S was measured using a Li-6400 infrared gas analyser (Li-Cor Biosciences, Lincoln, NE, USA) equipped with a Li-6400-09 soil efflux chamber (Fig. 5.1). The maximum difference between mean high and low daily temperatures was 2.6 º C but was less than 1 º C for most of the sampling periods. Exclusion of rainfall removed the impact of precipitation events on the results. Changes in root biomass and autotrophic respiration may have changed over the period, but those differences are expected to be minor.

5.2.2 Root decomposition

I used roots collected from a common pasture exposed to ambient CO_2 and precipitation to estimate decomposition rates under three levels of CO_2 and three irrigation treatments. Roots

for the decomposition experiment were excavated from 0 – 10 cm in the *L. perenne*-dominated paddock surrounding the TasFACE2 experiment. Roots were removed from soil clods and were soaked for 24 h in a 5% sodium hexametaphosphate solution (an aggregate dispersal agent) then were rinsed thoroughly in deionized water. Dead roots were separated from live roots based on colour and consistency and were discarded along with aboveground plant material. Live root material was dried to constant weight at 50º C. I put approximately 1.2 g dried roots into fiberglass packets (6 x 4 cm) with a mesh size of approximately 150 µm. On 9 September 2016, I installed litter bags into moist soil by first making a narrow, vertical cut in the soil surface to a depth of approximately 5 cm. One litter bag was installed in each cut with a total of 3 bags installed in each sector. One litter bag from each sector was collected on 11 November 2016, 21 February 2017 and 28 June 2017. This corresponds to deployment periods of 63, 164 and 291 d respectively. I soaked roots for 24 h in 5% sodium hexametaphosphate before rinsing with deionized water. Root material from the litter bags was dried at 50º C to constant weight before being weighed. I used an elemental analyser (Perkin Elmer, Melbourne, VIC, Australia) to measure C and N content of the root material from the initial deployment and from the final collection.

5.2.3 Statistical analysis

A linear mixed effects model with repeated measures was used to analyse the impact of experimental factors on R_S using the lme4 package (Bates et al. 2015) in R version 3.5.0 (R Core Team 2018). $[CO_2]$, irrigation, $[CO_2] \times$ irrigation and the $[CO_2] \times$ irrigation $\times$ month interaction term were all discrete variables included as fixed effects. As random effects, I nested sector number in block in a random intercept model. P-values were determined through the Likelihood Ratio Test of the full model compared to the full model with a single effect removed. Model selection was performed using the Akaike Information Criterion (AICc). Models containing irrigation and the $[CO_2] \times$ irrigation and $[CO_2] \times$ irrigation $\times$

month interaction terms were dropped from the final model due to P-values > 0.05 and AICΔ > 2.0. Soil temperature at 20 cm depth and percent water-filled pore space (WFPS) from 0 – 5 cm were tested as potential covariates but were not included in the final model due to non-linear associations with R_S. I estimated marginal means and 95% confidence intervals for [CO_2] and irrigation using the R package emmeans (Lenth 2018). Mass loss from the litter bag experiment was analysed using repeated measures ANOVA to identify effects of [CO_2] and irrigation. A post hoc pairwise comparison (Tukey HSD) was performed to identify differences between treatment groups. Visual inspection of residuals did not indicate deviations from homoscedasticity or normality.

5.3 Results

R_S rates varied considerably with a mean of 2.1 ± 0.2 µmol CO_2-C m^{-2} s^{-2} released in May to a mean of 6.6 ± 0.5 µmol CO_2-C m^{-2} s^{-2} released in November (Fig. 5.2). There was little variation in R_s between spring (September and November) and summer month (January), with a mean of 5.9 ± 0.3 µmol CO_2-C m^{-2} s^{-2} in spring to 4.4 ± 0.2 µmol CO_2-C m^{-2} s^{-2} in summer (Fig. 5.2). R_S under eCO_2 was ~75% higher on average relative to 400 µmol CO_2 mol^{-1} but no differences in R_S were present between 475 µmol CO_2 mol^{-1} and 550 µmol CO_2 mol^{-1} (Fig. 5.2). Importantly, the fact that R_S was increased in soils exposed to 475 µmol CO_2 mol^{-1} but was not increased further under 550 µmol CO_2 mol^{-1} demonstrates that the response of R_S to [CO_2] is non-linear between 400 µmol CO_2 mol^{-1} and 550 µmol CO_2 mol^{-1} in this grassland. Cumulative C released from the soil over the duration of the experiment was substantially higher under eCO_2, though there were no differences between 550 µmol CO_2 mol^{-1} and 475 µmol CO_2 (Fig. 5.3). The CO_2 effect on R_S varied between months with the smallest percent increase of 38% (effect size 0.4) occurring in May while the largest of 113% (effect size 3.8) occurred in November (Figs. 5.2, S1). Thus, spring was the time of highest R_S and the time of greatest CO_2 effect. The comparison of a linear model containing [CO_2] as a fixed factor and

soil temperature as a covariate to a model without soil temperature demonstrated soil temperature did not have a significant impact on R_S (F = 0.02, P = 0.9). Soil water content was altered substantially by the irrigation treatments (Table 5.1) but this did not translate into a significant impact on R_S (Fig. 5.2). Mean soil water content was reduced at the soil surface in *limit* sectors relative to other treatments by 13% WFPS on average (Table 5.2). The largest impact of irrigation on soil water content occurred in November, during which WFPS in *sufficient* and *excess* sectors was approximately double that measured in *limit* sectors under eCO_2 (Table 5.2). However, the size of this effect varied substantially between months with no effect of irrigation in September (Tables 5.1 and 5.2). Over winter months (June to August), irrigation volume was consistent between treatments (Table 2.1). Thus, differences in soil water content due to irrigation lagged following adjustment to spring treatments in September (Table 5.1).

Interestingly, the effect of soil moisture on R_S was non-linear across the wide range of soil water content measured throughout the experiment (Fig. 5.4). R_S was stimulated under eCO_2 relative to 400 µmol CO_2 mol^{-1}, an effect which was consistent with soil water content (Fig. 5.4). eCO_2 increased soil water content in all months (Tables 5.1 and 5.2), but again the effect was non-linear. WFPS was similar in plots under 475 and 550 µmol CO_2 mol^{-1} (Tables 5.1 and 5.2). Mean daily soil temperature increased over time from an average of 11.7 º C in autumn to 19.5 º C in summer (Table S.2). Soil temperature was independent of [CO_2] (χ^2 (2) = 0.2, P > 0.9) and irrigation (χ^2 (2) = 1.6, P > 0.5) throughout the experiment. Similar to the association between R_S and WFPS, R_S was consistently higher under eCO_2 compared to 400

1215 μmol CO_2 mol^{-1} over a wide range of soil temperatures (Fig. 5.4). There was a non-linear
1216 relationship between R_S and temperature with the highest fluxes occurring at the intermediate
1217 soil temperature of 15 º C (Fig. 5.4). This association may be due in part to increased plant
1218 water requirements in summer months when temperatures were highest (R^2 = 0.30, $F_{1,238}$ =
1219 104, P < 0.0001; Fig. 5.4).
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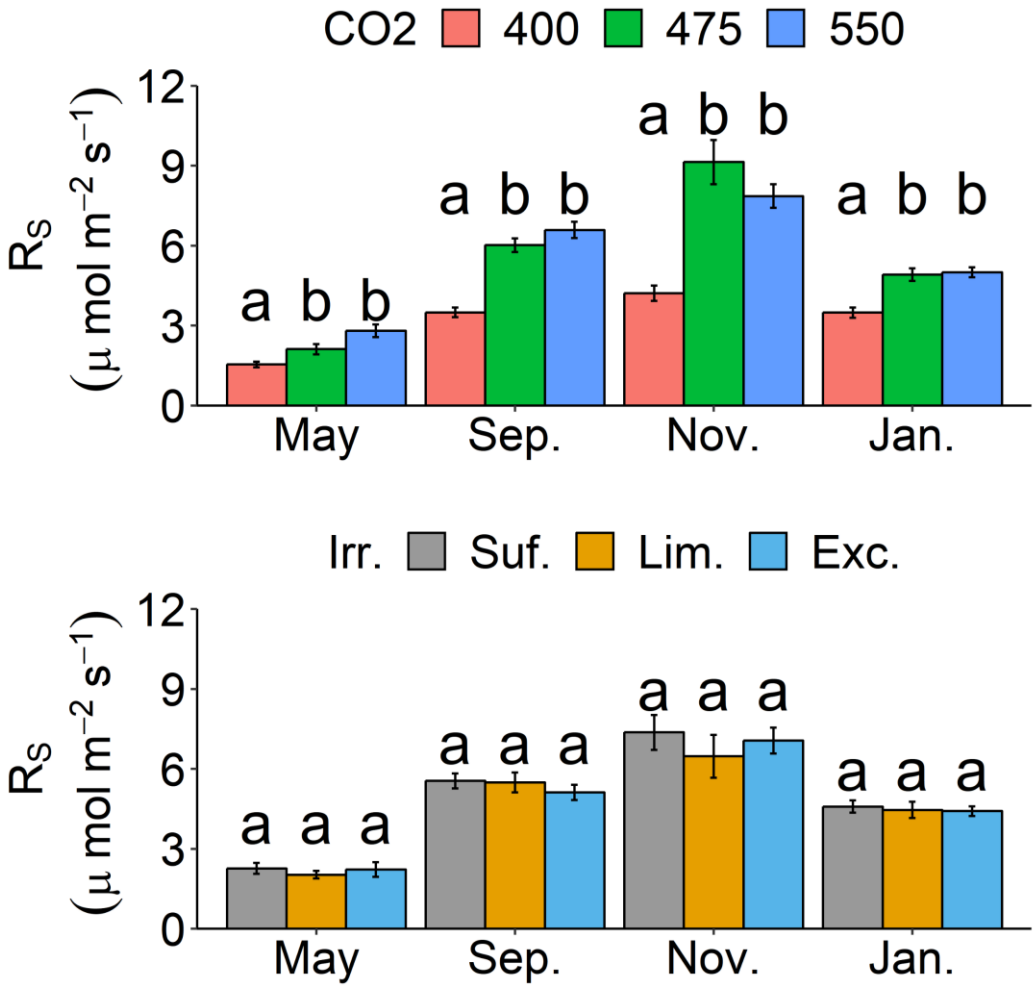

Figure 5.2. The impact of CO_2 level (top) and irrigation treatment (bottom) on soil respiration (R_S) from the TasFACE2 experiment. Each plot was exposed to one CO_2 level (400, 475 and 550 µmol CO_2 mol^{-1}) and one irrigation treatment (*sufficient*, *limit* and *excess*). R_S was measured over 14-d sampling campaigns in May 2016 (autumn), September 2016 (spring), November 2016 (spring) and January 2017 (summer). Those means with the same letter within a month are not significantly different (P > 0.05).

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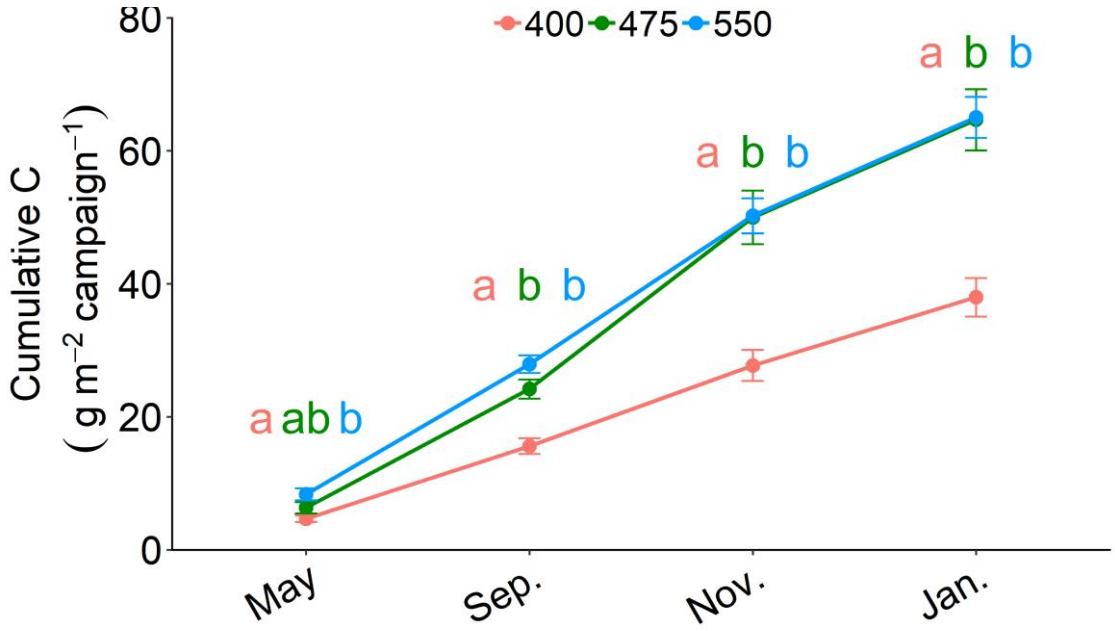

Figure 5.3. The impact of CO_2 level (400, 475 and 550 µmol CO_2 mol^{-1}) on cumulative C (g m^{-2} campaign $^{-1}$) from the TasFACE2 experiment from May 2016 to January 2017. Soil respiration (R_S) measurements were recorded daily over a 3-d campaign for each month. C released was estimated for each day of the campaign based on the daily average, which was then used to calculate cumulative C released. Those means with the same letter within a month are not significantly different (P > 0.05).

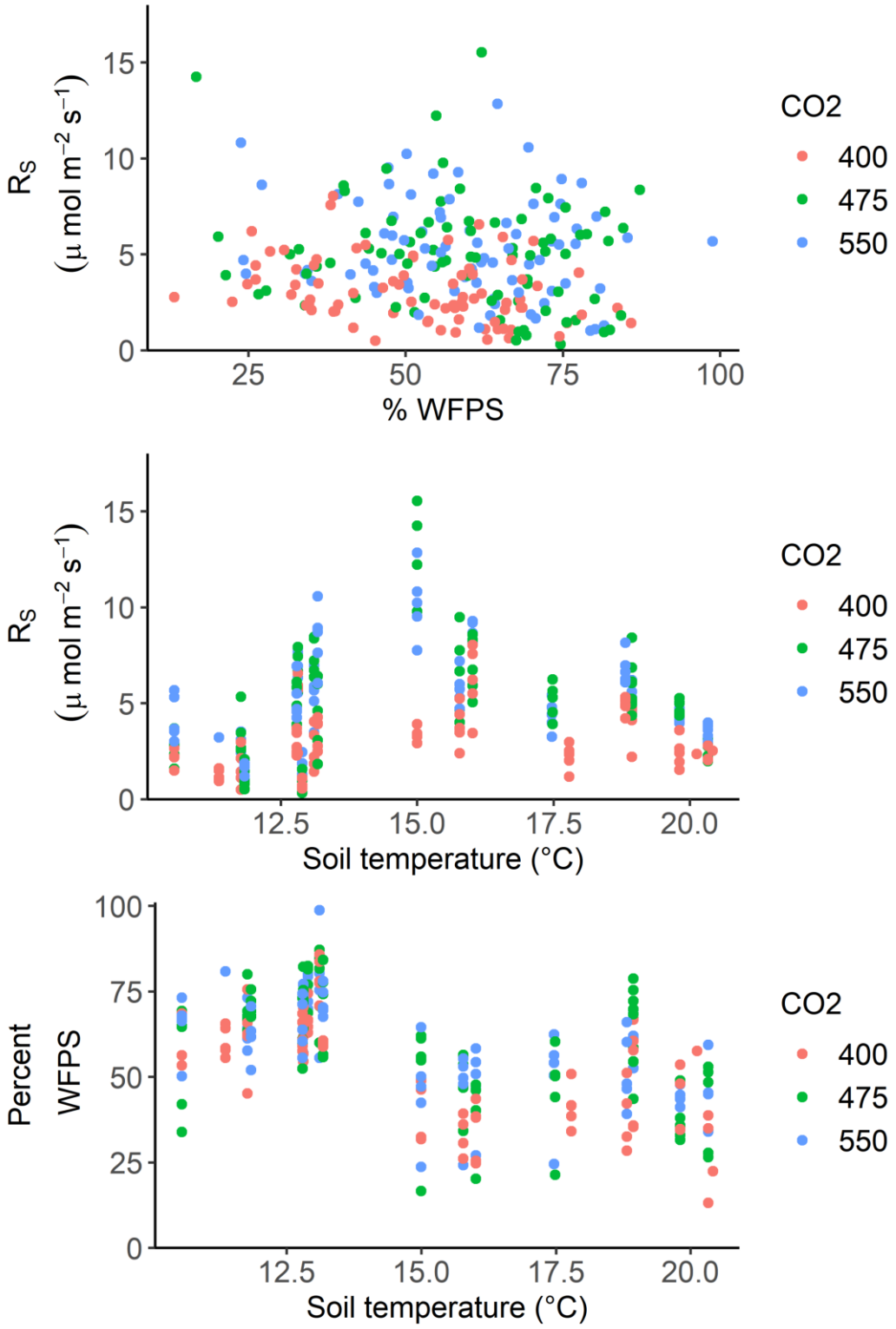

Figure 5.4. The association between soil respiration (R_S) and percent water-filled pore space (%WFPS; top) from 0-5 cm and soil temperature (middle) measured at 20 cm. The association between %WFPS and temperature (bottom) is also presented. Data were collected during 14-d R_S measurement campaigns between May 2016 and January 2017 from the TasFACE2 pasture.

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Table 5.1. Estimated effect of [CO_2] and irrigation on percent water-filled pore space (WFPS). Coefficients and SE were estimated using a linear mixed effects model containing [CO_2] (400, 475 and 550 µmol CO_2 mol^{-1}) and irrigation (*sufficient, limit* and *excess*) as fixed factors and block as a random effect. Irrigation was dropped from the final model in September (P > 0.1). The lower and upper limits of the 95% confidence interval are reported. P-values were calculated with the Likelihood-Ratio Test. Bold text indicates P value ≤ 0.05. Underline indicates 0.1 ≥ P value > 0.05.

Month	Variable	Coef.	SE	L. CI.	U. CI.	Chi sq.	df	P-value
May	Intercept	65.2	3.2	58.3	72.1	-	-	-
	CO2 [475]	4.8	2.3	0.3	9.3	17.8	2	<u>0.06</u>
	CO2 [550]	4.7	2.3	0.2	9.2			
	Irr. *Limit*	-7.0	2.5	-12.1	-2.0	8.4	2	**<0.001**
	Irr. *Excess*	1.9	2.5	-3.1	7.0			
	Random effects standard deviation: block = 4.1; residuals = 7.2							
Sep.	Intercept	65.7	3.2	58.1	73.3	-	-	-
	CO2 [475]	4.6	2.7	-0.8	9.9	5.9	2	**0.05**
	CO2 [550]	6.3	2.7	1.0	11.6			
	Irr. *Limit*	-	-	-	-	2.7	2	0.3
	Irr. *Excess*	-	-	-	-			
	Random effects standard deviation: block = 5.2; residuals = 8.5							
Nov.	Intercept	39.8	2.6	34.7	45.0	-	-	-
	CO2 [475]	8.7	3.0	2.7	14.7	11.9	2	**0.003**
	CO2 [550]	10.0	3.0	4.0	15.9			
	Irr. *Limit*	-15.3	3.3	-21.9	-8.6	22.3	2	**<0.0001**
	Irr. *Excess*	1.2	2.7	-4.3	6.6			
	Random effects standard deviation: block < 1.0; residuals = 9.5							
Jan.	Intercept	45.2	5.0	33.5	56.9	-	-	-
	CO2 [475]	9.5	3.2	3.1	15.9	9.1	2	**0.01**
	CO2 [550]	7.8	3.2	1.4	14.1			
	Irr. *Limit*	-9.9	3.6	-17.0	-2.7	7.3	2	**0.03**
	Irr. *Excess*	-2.1	2.9	-7.9	3.8			
	Random effects standard deviation: block = 8.3; residuals = 10.1							

Table 5.2. Mean percent water-filled pore space (WFPS) ± SE in soils from 0 - 5 cm in sectors exposed to three levels of CO_2 (400, 475 and 550 µmol CO_2 mol^{-1}) and three irrigation volumes (*sufficient*, *limit*, and *excess*). Data were averaged over 14-d campaigns when soil respiration (R_S) was measured in May 2016, September 2016, November 2016 and January 2017.

		WFPS					
		400		475		550	
Month	Irrigation	µmol CO_2 mol^{-1}		µmol CO_2 mol^{-1}		µmol CO_2 mol^{-1}	
May	*Sufficient*	80.3	± 1.6	87.7	± 1.7	84.1	± 2.8
	Limit	66.4	± 1.9	72.8	± 4.3	75.9	± 2.0
	Excess	79.1	± 0.5	83.1	± 1.7	81.9	± 3.2
Sep.	*Sufficient*	75.8	± 2.6	85.1	± 3.3	88.5	± 2.0
	Limit	79.3	± 2.2	82.0	± 4.5	72.5	± 4.3
	Excess	80.1	± 3.3	83.0	± 3.3	91.3	± 3.2
Nov.	*Sufficient*	42.3	± 2.9	56.4	± 2.3	56.3	± 1.7
	Limit	28.3	± 2.2	26.4	± 5.4	27.9	± 4.3
	Excess	38.2	± 1.5	56.6	± 3.3	59.4	± 3.0
Jan.	*Sufficient*	50.2	± 5.0	56.9	± 4.7	54.8	± 3.2
	Limit	33.9	± 4.3	45.7	± 6.4	53.1	± 6.1
	Excess	47.0	± 3.0	66.7	± 3.8	58.6	± 3.0

Root litter bags lost an average of 63 ± 1.6 % of initial root mass over the 291-d period and there was a substantial effect of [CO_2] on the rate of mass loss ($F_{2, 38}$ = 10.7, P = 0.0002; Fig. 5.5). However, the CO_2-effect on root mass loss was consistent between 475 and 550 µmol CO_2 mol^{-1} (adj. P > 0.5), meaning that the effect on root decomposition of elevating the [CO_2] was the same whether the increase was 75 or 150 µmol CO_2 mol^{-1}. At the end of the 291-d experiment, 34 ± 2.5 % of the original mass remained in the litter bags under 475 and 550 µmol CO_2 mol^{-1} plots while 43 ± 2.8 % remained in 400 µmol CO_2 mol^{-1} plots ($F_{2, 43}$ = 3.5, P = 0.04; Fig. 5.5), so eCO_2 stimulated the litter decomposition rate by 16%. The CO_2 effect on mass loss varied over time, with approximately 45% loss at 63 d under 475 and 550 µmol CO_2 mol^{-1} compared to approximately 25% loss under ambient CO_2 over the same time ($F_{2, 42}$ = 5.4, P = 0.008; Fig. 5.5). There was a slight increase in root mass at 164 d relative to 63 d, though this difference was not significant for 400 µmol CO_2 mol^{-1} (adj. P > 0.5), 475 µmol CO_2 mol^{-1} (adj. P = 0.3) or 550 µmol CO_2 mol^{-1} (adj. P = 0.06). There was no effect of

irrigation treatment on mass loss ($F_{2, 38}$ = 0.7, P > 0.5) nor was there a CO_2 × irrigation interaction ($F_{2, 38}$ = 0.7, P > 0.5). Although the C:N of the root material decreased from 28.6 in the initial material to 22.8 in the material collected at 291 d ($F_{3, 45}$ = 5.4, P = 0.003), this change was independent of CO_2 ($F_{2, 45}$ = 1.1, P = 0.3) and irrigation treatments ($F_{4, 45}$ = 0.5, P = 0.7).

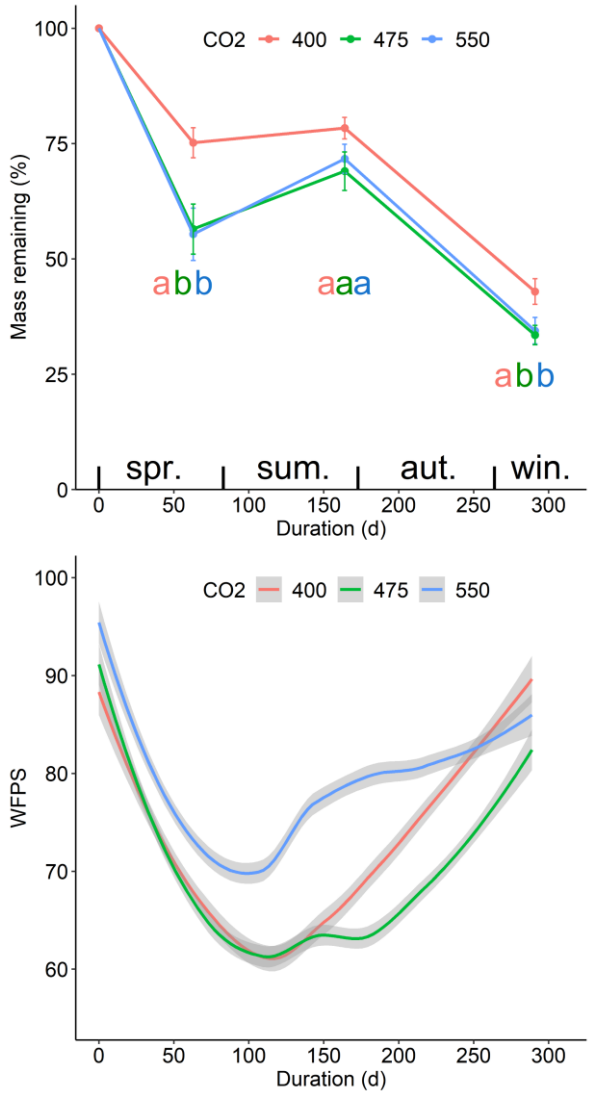

Figure 5.4. Impact of CO_2 level on percent mass loss of common pasture root samples (top) and percent water-filled pore space (WFPS) at 20 cm (bottom). Litter bags containing root material were deployed in plots on 9 September 2016 (spring) and remained in the soil through 27 June 2017 (winter). Litter bags were collected at 63 d, 164 d and 291 d. Those means with the same letter within a month are not significantly different (P > 0.05). Shading is the 95% confidence interval.

5.4 Discussion

A stimulatory effect of eCO_2 on the availability of C and water in the soil, due to the responses of the plants to the higher [CO_2], has been demonstrated previously to increase R_S and organic matter decomposition in grasslands with two levels of CO_2 (Adair et al. 2011, Selsted et al. 2012, Osanai et al. 2015, Ryan et al. 2015) but it is unclear whether these relationships increase linearly with increasing [CO_2]. Some species of C_3 grasses respond to increasing [CO_2] with linear increases in photosynthesis (Gill et al. 2002b) and water-use efficiency (Polley et al. 1993, Anderson et al. 2001b), suggesting that soil C input and mean soil moisture should also increase with [CO_2]. Because some factors causing the CO_2 effect on R_S are changes in C availability (Adair et al. 2011) and soil moisture (Pendall et al. 2003, Wan et al. 2007), it is likely a linear increase in these parameters with [CO_2] may drive a similar increase in R_S. In TasFACE2, I found R_S and root decomposition were accelerated under eCO_2 relative to ambient CO_2, but contrary to our expectations, both processes occurred at similar rates under 475 and 550 µmol CO_2 mol^{-1}. This suggests that the response of R_S to [CO_2] may be difficult to generalise due to the complex factors underlying this flux.

R_S may not respond to [CO_2] in a predictable manner due to the varied responses of autotrophs and heterotrophs to changes in C availability. Autotrophic respiration typically increases with root activity in grasslands due to the production of CO_2 during root growth and maintenance (Wan et al. 2007). Therefore, it is unsurprising R_S was highest in spring (Fig. 5.2) when root production was highest in this system (Fig. S.1). However, it is unclear how much of the spring stimulation was driven by autotrophic respiration. Root respiration can comprise between 16 to 58% of R_S in forests (Rodeghiero and Cescatti 2006) and between 10 to 90% in grasslands (Hanson et al. 2000). Given the importance of root activity on R_S, I expected a strong CO_2-effect on R_S due to the same effect on root production. However, the linear effect of [CO_2] on root production resulted in no similar linear increase in R_S with

1270 [CO_2]. This suggests that a nonlinear response in heterotrophic activity may have contributed
1271 to the lack of a linear response in R_S in this system.

1272 Rhizodeposition was undoubtedly increased under eCO_2 here given the impact of [CO_2] on
1273 root production (Fig. S1), which is generally associated with increased belowground C
1274 availability for microbes (Pendall et al. 2004, de Graaff et al. 2010). Heterotrophs were
1275 provided additional substrate via root exudation, a process which functions on a short time
1276 scale relative to root turnover and is strongly impacted by [CO_2] (Hungate et al. 1997b).
1277 Increased C input to soil likely increased the breakdown of soil organic matter by stimulating
1278 microbial enzyme activity (Phillips et al. 2011, Shahzad et al. 2015b). Although it is likely
1279 exudation rates increased linearly with [CO_2] (Hungate et al. 1997b), this may not have
1280 translated to linear increases in heterotrophic respiration due to physiological constraints of
1281 microorganisms. At high C:N of organic matter or low concentration of SOM, decomposer
1282 activity can be nonlinear due to the high resource investment necessary for enzyme
1283 production (German et al. 2011) and due to limitations on access to N (Allison et al. 2014).
1284 As such, the differences in soil C input from exudation between 475 and 550 μmol CO_2 mol^{-1}
1285 likely was insufficient to meet the threshold necessary for a linear decomposition rate
1286 (German et al. 2011). The roots in this study were dried and consequently may have had
1287 lower decomposition rates than equivalent live roots. As such, care should be taken when
1288 comparing these results to similar studies using live roots. The CO_2 effect on organic matter
1289 input to soils was likely reduced due to the length of time the site was exposed to eCO_2. Root
1290 turnover comprises a major source of belowground C and N (Iversen et al. 2008), yet root
1291 lifespan can exceed 1 y in grasslands (Trumbore and Gaudinski 2003). Thus, it likely will be
1292 several years before a CO_2-effect on organic matter inputs will impact soil heterotrophic
1293 respiration in a measurable way. Organic matter input rates may have been further impacted
1294 by site methods. Litterfall typically increases with [CO_2] through greater aboveground

biomass production (de Graaff et al. 2006), but routine clipping and removal of aboveground biomass eliminated these inputs from the soil C supply entirely in TasFACE2. However, due to the strong linear impact of [CO_2] on root production in this system (Fig. S1), the absence of aboveground litterfall may have weakened but not eliminated a CO_2 effect on heterotrophic respiration. Clipping may also have reduced root production as has been observed in other grasslands (Wilsey et al. 1997), though such an effect would be consistent between CO_2 treatments if it was present. Thus, R_S might have been lower in TasFACE2 relative to an equivalent unclipped grassland but such an effect would not greatly impact treatment effects on R_S. Root biomass in the decomposition experiment may have been overestimated due to inadvertent measurement of soil. This is expected to be minor given the detailed washing that occurred.

At the soil surface in TasFACE2, eCO_2 increased WFPS relative to plots exposed to ambient CO_2 regardless of whether soils were fumigated with 475 or 550 µmol CO_2 mol^{-1} (Table 5.2). The positive impact of eCO_2 on R_S persisted in 475 and 550 µmol CO_2 mol^{-1} and across a range of soil water content (Fig. 5.4). Importantly, this indicates that in this grassland, the association between [CO_2] and R_S is generalised and consistent regardless of water availability. Contrary to our hypothesis, these results demonstrate that the stimulation of R_S and decomposition under eCO_2 is largely unaffected by changes in plant-water relations in this grassland.

The impact of eCO_2 on WFPS is consistent with the responses observed broadly in systems with two CO_2 levels (Ainsworth and Rogers 2007, Leakey et al. 2009), but does not support the possibility of a linear relationship between soil moisture and [CO_2] (Polley et al. 1993). The water-saving response of C_3 grasses to [CO_2] may be insufficient in size to elicit detectable differences in soil water content between eCO_2 levels. Minor changes in WFPS are difficult to assess *in situ* as this metric can vary substantially over a small area and can cause

a high level of variation in R_S (Xu and Qi 2001). The disparity between WFPS measured at the soil surface and 20 cm depth emphasises high variability in soil water content (Fig. 5.5). For example, eCO_2 increased WFPS under 475 µmol CO_2 mol^{-1} and 550 µmol CO_2 mol^{-1} relative to 400 µmol CO_2 mol^{-1} at the soil surface but only increased WFPS under 550 µmol CO_2 mol^{-1} at 20 cm depth (Fig. 5.5). The majority of CO_2 produced via R_S occurs at the soil surface (Fang and Moncrieff 2005), thus manual moisture readings closely reflect soil conditions which would have affected this process.

This finding is further supported by the results of the irrigation treatments, which show a clear impact on WFPS over the duration of this study (Tables 5.1 and 5.2). The lack of difference in WFPS between irrigation treatments in September was due to carryover from the winter irrigation treatments, which provided equal water to all sectors from June through August (Table 2.1). In combination with low water uptake by plants in early spring, it is unsurprising WFPS in September was unchanged by irrigation. In general, the impact of irrigation treatment on WFPS was significant. For example, in November, WFPS in *sufficient* and *excess* treatments were approximately double the WFPS in *limit* sectors (Table 5.2). Despite such large differences in soil water availability, R_S was unaffected by irrigation treatments during any period of the experiment (Fig. 5.2). I hypothesized the CO_2 effect on R_S would increase in 'dry' soil conditions due to increased water-use efficiency as has been found in some instances (Pendall et al. 2003, Zhou et al. 2016). However, the analysis found no significant CO_2 × irrigation interaction. Although there was a positive response of R_S to CO_2, this effect was independent of soil water content (Fig 5.2). The strongest CO_2 effect on R_S as a result of increased soil moisture is observed in arid or semiarid grasslands, which have substantially lower water content than even in the driest periods in TasFACE2 (Pendall et al. 2003, Wei et al. 2016). Changes in soil water content mesic grasslands may not have a

large impact on R_S due to the relatively greater impact of substrate availability (Wan et al. 2007, Adair et al. 2011).

In conclusion, both R_S and decomposition of root material in the TasFACE2 experiment were accelerated by eCO_2 treatments but there were no differences between the two eCO_2 levels. This demonstrates that in some grasslands, the response of these processes to $[CO_2]$ is nonlinear. I expected these responses to be driven by plant-mediated shifts in C and water availability. However, R_S and decomposition remained similar across a wide range of soil water content, proving that C availability rather than water content was the primary factor driving the CO_2-effect on these processes in this grassland. Although climate models predict ecosystems will be affected by climate change through simultaneous shifts in soil water content and increases in $[CO_2]$, our findings demonstrate moderate shifts in water content in mesic soils may not impact R_S or root decomposition. However, the importance of $[CO_2]$ on soil C input and subsequently R_S and root decomposition is emphasized by the significant impact of $[CO_2]$ observed over the first year of fumigation, during which the only soil C inputs were derived from root sources such as exudation and turnover to a lesser extent. Our findings demonstrate the need to determine whether the nonlinear response of R_S and root decomposition to $[CO_2]$ persists with the inclusion of above- and belowground organic matter inputs and if this pattern changes over time.

6 GENERAL DISCUSSION

The overarching aim of this thesis was to assess the influence of future climate conditions on the biogeochemical processes that regulate soil C storage, productivity, and climate change feedbacks in a managed pasture. In order to do that, I specifically investigated the impact of [CO_2] on soil respiration (R_S) and root decomposition, availability of soil mineral N and the emission of the potent greenhouse gas N_2O. The results show that levels of soil mineral N essential for primary production declined as [CO_2] increased due in part to plant immobilisation of N and possibly production of N_2 and NO. N_2O emissions from the pasture were very high following urea application regardless of [CO_2]. Under eCO_2, N_2O emissions were never higher than emissions under ambient CO_2 and were often substantially lower, due to declines in soil NO_3^-. R_S, root decomposition and mineralisation of soil organic C were increased under eCO_2 with only variable changes in biomass production to [CO_2]. This suggests future atmospheric [CO_2] may accelerate C cycling and reduce C storage in pasture soils. My findings indicate [CO_2] may reduce N_2O emissions from temperate pastures and reduce availability of mineral N for plant production. Through the independent control of [CO_2] and water supply, I determined that the responses in C and N cycling with [CO_2] were driven almost entirely by the CO_2-effect on root production and belowground C input rather than an indirect CO_2-effect on soil water content. Plant responses to [CO_2] may constrain the emission of N_2O in some pastures, but nitrogenous fertiliser application remains the strongest influence on the emission of this gas. The following synthesis integrates the impacts of global change treatments on C and N cycling to describe key drivers of ecological change and directions for future studies.

6.1 *Processes driving CO_2 emissions*

Box 1: Summary of eCO_2 effect on C cycle processes

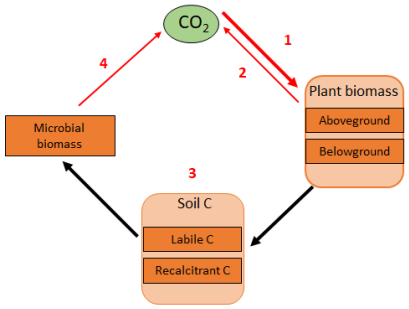

Figure 6.1. A conceptual diagram representing the processes influencing CO_2 emissions from soils. Red arrows indicate processes that were measured in this study. Bold arrows indicate processes affected by eCO_2 based on the literature. Under eCO_2, the rate at which C moved between pools within the C cycle was accelerated due primarily to greater organic C inputs to soil microbes. Aboveground production decreased with [CO_2] (Fig. 6.1 Process 1), while belowground biomass production was highest under 550 µmol CO_2 mol^{-1} but was similar between 475 and 400 µmol CO_2 mol^{-1}. Substantial root growth under 550 µmol CO_2 mol^{-1} resulted in higher total biomass than the other CO_2 levels, which had similar total biomass. As gross primary production is the strongest predictor of autotrophic respiration (Fig. 6.1 Process 2), it was expected R_S would be highest under 550 µmol CO_2 mol^{-1} with no increase under 475 µmol CO_2 mol^{-1}. However, R_S was higher under eCO_2 but was similar between 475 and 550 µmol CO_2 mol^{-1}, suggesting the possibility that heterotrophic respiration rates (Fig. 6.1 Process 4) exceeded autotrophic respiration here. However, without partitioning the two sources this remains unclear. Under eCO_2, root decomposition was also increased as was the mineralisation of soil organic C, demonstrating stimulated microbial activity. Independent control of water supply and [CO_2] indicated that these processes were driven by the CO_2-effect on belowground C input (Fig. 6.1 Process 3) rather than the indirect CO_2-effect on soil water content. As aboveground biomass was removed, C inputs were entirely from belowground inputs, emphasising the importance of roots on C dynamics here. Belowground C input may have been driven by the CO_2-effect on root exudation seeing as aboveground biomass was harvested and the CO_2-effect on root turnover was probably minor here. The strong impact of [CO_2] on C cycling suggests the importance of root exudates on microbially-mediated processes and climate change feedbacks.

C mineralisation and root decomposition were accelerated under eCO_2 but there were no significant differences between these process rates in soils under 475 and those under 550 µmol CO_2 mol^{-1}. Under eCO_2, CO_2 emissions from soil via R_S were increased by between 38% and 113%, root decomposition was increased by 16% and soil organic C stocks were also reduced, compared to control plots. These changes were driven by shifts in plant productivity, which stimulated microbial activity through greater belowground C input.

Plant production is the primary input of C into terrestrial ecosystems (Fig. 6.1 Process 1) and therefore affects belowground C input and root activity. As production increases with [CO_2], the supply of C provided to decomposers as well as the respiration of roots will also be affected by [CO_2]. Organic C contained in plant biomass was estimated by measuring aboveground and belowground production via routine harvesting and root cores respectively. Aboveground biomass production was reduced under eCO_2 relative to ambient CO_2, with a CO_2-effect of -17% under 475 µmol CO_2 mol^{-1} and -26% under 550 µmol CO_2 mol^{-1} (Fig. S1). Conversely, belowground biomass was increased by 9% under 475 µmol CO_2 mol^{-1} and by 30% under 550 µmol CO_2 mol^{-1} relative to ambient CO_2 (Fig. S1). Total production increased under 550 µmol CO_2 mol^{-1} relative to 400 µmol CO_2 mol^{-1} due to greater root production but no similar effect was present in 475 µmol CO_2 mol^{-1}. CO_2 is released from roots as a by-product of root production and maintenance via autotrophic respiration (Fig. 6.1 Process 2). The rate at which roots respire is associated with environmental conditions and can be related to total plant biomass as this determines photosynthate supply (Gomez-Casanovas et al. 2012). [CO_2] typically increases total plant biomass and increases photosynthate supply allocated to roots (de Graaff et al. 2006), which may further drive autotrophic respiration through additional root activity. Based on total plant production alone, autotrophic respiration likely was highest under 550 µmol CO_2 mol^{-1} as this CO_2 treatment had the most biomass (Fig. S1). I expected no difference between 400 and 475 µmol CO_2

mol^{-1} as total plant production was similar between the two treatments. Interestingly, R_S was similar between the two eCO_2 levels on all sampling dates and was substantially higher than in plots at 400 µmol CO_2 mol^{-1} (Fig. 5.2). R_S was highest in spring when production was greatest, though R_S remained similar between eCO_2 levels despite a substantial increase in production under 550 µmol CO_2 mol^{-1} during this time (Fig. S1). Autotrophic respiration can be affected by temperature, moisture and N supply, though gross primary production is considered the strongest driver by far (Hopkins et al. 2013). Of course, autotrophic respiration comprises just one portion of this flux and consequently may not be representative of R_S. The contribution to R_S from either source can vary from 10 to 90% (Hanson et al. 2000) and thus could be dominated by heterotrophic sources in this system. Seeing as root decomposition was similarly increased under eCO_2 but did not differ between 475 and 550 µmol CO_2 mol^{-1} suggests the heterotrophic respiratory component responded similarly. I am unable to ascertain if this is the case, however, as the sources of CO_2 from autotrophic and heterotrophic sources were not partitioned in this experiment.

Plant production has a strong influence on the organic matter supplied to soil microbes (Fig. 6.1 Process 3) and thus can impact the microbial community and heterotrophic respiration (Fig. 6.1 Process 4). In this system, C input to soil microbes from plants was due entirely to root-derived inputs as aboveground biomass was removed. Soil heterotrophs produce CO_2 during the oxidation of soil organic matter and consequently are dependent on organic inputs from plant and microbial necromass as well as from root exudates. Rhizodeposition was the only plant-derived C source given that aboveground biomass was removed by harvesting. Therefore, root dynamics may have had a strong effect on heterotroph activity. Under eCO_2, the root decomposition rate was 16% higher than ambient rates, indicating C and N in sloughed roots were rapidly decomposed (Fig. 5.5). Further, eCO_2 accelerates root turnover in some cases, suggesting a larger pool of root necromass coupled with faster decomposition

could lead to increased organic C availability under eCO_2 (Madhu and Hatfield 2013). However, it is unclear to what extent the CO_2-effect on root turnover may have altered soil organic matter supply in this young experimental site given root lifespan can exceed 1 y (Trumbore and Gaudinski 2003). On the other hand, root exudation likely was a major factor influencing microbially-mediated decomposition as this process is sensitive to photosynthate supply and functions on short time scales (Walker et al. 2003). Root exudates provide labile C to soil microbes and have been shown to induce accelerated microbially-mediated decomposition of soil organic matter via the rhizosphere priming effect (Kuzyakov 2002b). Aboveground biomass and total biomass have the strongest association with belowground C input via root exudation (Huo et al. 2017), which suggests root exudation was probably highest under 550 µmol CO_2 mol^{-1} and absent entirely under 475 µmol CO_2 mol^{-1} due to the CO_2-effect on total production (Fig. S1). However, based on the reduction in soil C under eCO_2 (Fig. 3.3), it appears the rhizosphere priming effect was present to some extent in both 475 and 550 µmol CO_2 mol^{-1} despite the discrepancy in total biomass produced. Furthermore, the decline in soil organic C was similar between 475 and 550 µmol CO_2 mol^{-1} (Fig. 3.3), indicating that the accelerated rate of soil organic matter decomposition induced by [CO_2] was similar between 475 and 550 µmol CO_2 mol^{-1}. This finding is supported by the emission of CO_2 via R_S, which was stimulated under eCO_2 but did not differ between 475 and 550 µmol CO_2 mol^{-1} (Fig. 5.2). Future studies should include measures of soil microbial biomass C as this will demonstrate to what extent C is immobilised in microbial pools. Additionally, measurement of soil C fractions will help to assess which pools decreased in size with [CO_2]. These findings emphasize the importance of root exudates on microbial processes given these inputs appear to have driven most changes in C turnover in this system.

Through independent control of [CO_2] and soil water supply, this study demonstrated that R_S in this grassland was influenced by a CO_2-effect on plant production, belowground organic

matter input or a combination of the two and was unaffected by the indirect CO_2-effect on soil water content (Figs. 5.2 and 5.4). The impact of WFPS, irrigation and the CO_2 × irrigation interaction effects on the emission of CO_2 were explicitly tested but the results demonstrated that R_s was not significantly affected by watering treatments and neither was the CO_2 effect on R_s (Figs. 5.2 and 5.4). The negligible effect of soil water content here may be due in part to the small response of microbial activity to minor changes in WFPS in mesic soils. Between 30 to 70% WFPS, changes in soil water content have little impact on heterotrophic respiration compared to the relatively large impact of changes in soil water content on heterotrophic respiration above or below these optimal conditions (Moyano et al. 2013). Over the duration of this study, rhizosphere soil water content was typically between 25-80% WFPS and was rarely beyond this range due to twice-weekly irrigation events and exclusion of rainfall (Fig. 5.4). The absence of an indirect CO_2-effect on R_S due to soil moisture may be due partly to the high frequency of watering events which maintained mesic soil water content levels. An equivalent pasture exposed to episodic, high-volume rainfall events and extended periods without water would have a greater range of WFPS and potentially very different CO_2 emissions. The water-saving effect of CO_2 is typically strongest in soils that are dry (Liu et al. 2009) or saturated (Wan et al. 2007) as plant-mediated shifts in water content alleviate microbial water constraints or lower soil oxygen content respectively. Thus, a shift in soil water content mediated by plants under eCO_2 may yield a stronger CO_2 emission response in pastures exposed to natural precipitation compared to irrigated pastures, which are typically maintained at mesic soil conditions to maximise pasture growth.

6.2 *Processes driving N_2O emissions*

Box 2: Summary of eCO_2 effect on N cycle processes

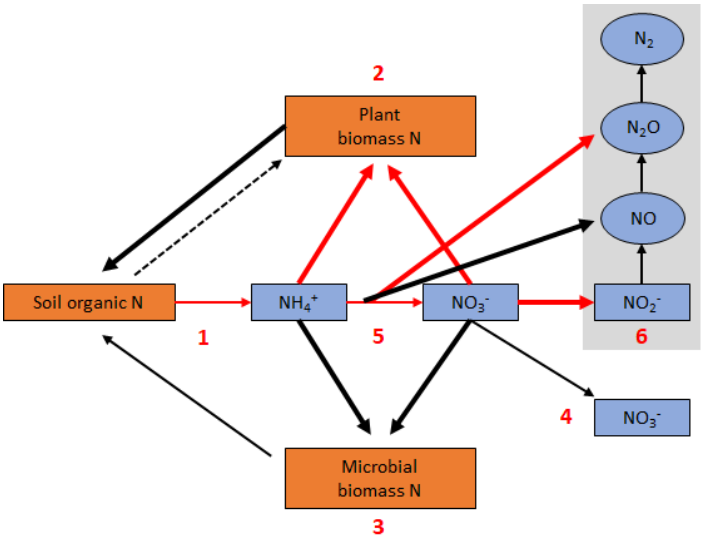

Figure 6.2. A conceptual diagram representing the relevant processes driving the emission of N_2O. Red arrows indicate processes that were measured in this study. Bold arrows indicate processes affected by eCO_2 based on the literature. Emissions can be produced through nitrification (Fig. 6.2 Process 5) or denitrification (Fig. 6.2 Process 6) and therefore require NH_4^+ or oxidised N respectively. In TasFACE2, N_2O emissions were never higher under eCO_2 and were usually lower than ambient emissions. Interestingly, emissions were sensitive to soil NO_3^- levels (Table 4.3) but were unaffected by soil water content. As such, factors affecting mineral N availability had the strongest impact on N_2O emissions. Total plant N (Fig. 6.2 Process 2) was increased under 550 µmol CO_2 mol^{-1} and, to a lesser extent, 475 µmol CO_2 mol^{-1}, both due entirely to root growth (Fig. 4.4). This effect corresponded with a substantial decline in soil NO_3^- under 550 µmol CO_2 mol^{-1} and a significant but smaller decline under 475 µmol CO_2 mol^{-1}.This effectively constrained denitrification through NO_3^- availability (Fig. 6.2 Process 6). Nitrification (Fig. 6.2 Process 5) was probably a large source of N_2O emissions following urea application when NH_4^+ availability was high (Table 3.2). Nitrifier denitrification may also have been a contributor as it is insensitive to soil water content. Declines in soil N suggest microbially-mediated mineralisation rates *in situ* were probably stimulated under eCO_2 via greater input of labile C. The reduction in soil N indicates microbial biomass N (Fig. 6.2 Process 3) was probably not increased with [CO_2], therefore declines in mineral N were primarily caused by plant N immobilisation.

Nitrogen availability was shown to be, by far, the most important factor influencing N_2O emissions from this pasture. Extremely high rates of N_2O emissions followed the application of urea in all treatment groups. 98% of estimated emissions over 30 d occurred within 1 week of urea application and accounted for an average of ~20% of the 4.4 g urea-N applied to each 0.5 m^2 sector. Emissions were much higher than the regional average for managed temperate pastures in Australia and New Zealand, in which between 0 and 1.56% of applied urea-N is released as N_2O-N (Luo et al. 2007). However, emissions comparable to TasFACE2 have been observed in similar pastures following fertiliser application (Clayton 1994). In TasFACE2, fertiliser application rate may have driven this massive increase given fertiliser is the single strongest factor influencing soil N_2O emissions (Shcherbak et al. 2014). *L. perenne* biomass yields increase nearly proportionally with N application up to approximately 1.5 kg N ha^{-1} d^{-1}, beyond which biomass yields and plant N uptake are relatively unchanged (Rawnsley et al. 2014). Therefore, N addition in excess of 1.5 kg N ha^{-1} d^{-1} in pastures could result in high levels of available mineral N for denitrifiers. Many studies similar to this one applied between 0 and 1.5 kg N ha^{-1} d^{-1} and had substantially lower emissions (Baggs et al. 2003b, Luo et al. 2007, Kammann et al. 2008). Undoubtedly, the 2.3 kg N ha^{-1} d^{-1} applied to TasFACE2 exceeded plant N demand and resulted in substantial denitrification. Fertiliser N form can also affect these emissions. Additionally, soil water content was a strong predictor of N_2O emissions in similar pastures as numerous studies reported high emissions following rainfall (Baggs et al. 2003b, Luo et al. 2007, Kammann et al. 2008). Soil water content in TasFACE2 was maintained at relatively constant levels through twice-weekly irrigation events. Therefore, anaerobic hotspots may have been prevalent in this pasture, potentially leading to high emissions here particularly given the abundance of NO_3^-. [CO_2] also had a strong suppressive effect on N_2O emissions here. Under ambient CO_2, 39% of applied urea-N was released as N_2O from soils while just 11% of applied urea-N was released as N_2O under

both 475 and 550 µmol CO_2 mol^{-1}. This dramatic effect of eCO_2 on N_2O emissions was driven by strong plant responses, which altered C and N availability for soil microbes. The TasFACE2 emissions presented here are likely overestimates, given these calculations were performed by extrapolating daily, 1-hr flux estimates to 24-h figures. Actual emissions undoubtedly varied over time due to diurnal changes in soil temperature and decreasing N availability. Nevertheless, the patterns observed here are unlikely to be altered by greater sampling frequency or more conservative calculations.

Soil mineral N in the form of NH_4^+ and NO_3^- are the molecules upon which production in terrestrial systems are typically limited and influence N_2O production via microbially-mediated nitrification and denitrification. The transformation of soil organic matter N derived from plant and microbial necromass into inorganic forms via mineralisation regulates the input of inorganic N (Fig. 6.2 Process 1). In TasFACE2, the organic N pool was reduced substantially under eCO_2 in autumn and winter though this effect was significant only under 550 µmol CO_2 mol^{-1} (Fig. 3.3 c and d). This was likely caused by the rhizosphere priming effect, the same mechanism which reduced soil organic C under eCO_2 in this study. Under eCO_2, belowground labile C input via exudation was increased, which likely stimulated microbial activity and consequently mineralisation rates. This effect has been shown to be an important mechanism driving mineral N supply in forests (Finzi et al. 2015) and grasslands (Zhu et al. 2014). Interestingly, a laboratory incubation of TasFACE2 soils indicated that there were no effects of [CO_2] on potential net mineralisation rates (Table 3.2). Reduction in soil N can be driven by declines in organic matter input from plants and microbes, though such an effect would require years to develop. The primary mechanism for accelerated mineralisation under eCO_2 is labile C inputs via root exudation, a C source which was not included in these incubations. Therefore, the results of the incubation assay likely do not accurately reflect the increased inorganic N input in field conditions. Therefore, future studies

should consider *in situ* estimates of N transformation processes as this approach may capture the important role of root exudates on mineral N input.

There was a strong decline in mineral N availability with [CO_2] (Figs. 3.1 and 3.2). The Progressive Nitrogen Limitation theory attributes declines in soil mineral N under eCO_2 to increased immobilisation of N in plant and microbial biomass as well as stable soil organic matter pools (Luo et al. 2004). In this study, the decline in soil mineral N with [CO_2] was driven in part by CO_2-mediated stimulation of plant N immobilisation (Fig. 6.2 Process 2) but likely was unaffected by microbial N immobilisation (Fig. 6.2 Process 3). Total N contained in plant biomass was increased under 550 µmol CO_2 mol^{-1} due primarily to root production (Figs. 4.4, S1). The stimulation of root production was modest under 475 µmol CO_2 mol^{-1} and consequently the total N contained in plants under 475 µmol CO_2 mol^{-1} was much lower than the N contained in plants under 550 µmol CO_2 mol^{-1}. There was no change in the C:N ratio of plant tissues with [CO_2] as is found in some other FACE experiments (Gifford et al. 2000), thus total N was closely associated with biomass production in this system and was unaltered by a CO_2-effect on tissue N content. Additionally, plant tissue C:N can influence soil C:N, thereby altering microbial N immobilisation rates. Microbial immobilisation of N has been shown to increase N contained in microbial biomass, resulting in a modest sink of N (de Graaff et al. 2006). As there was no CO_2-effect on plant tissue C:N, this had no effect on soil processes here. Thus, findings from this study suggest that microbial biomass N was unaffected by [CO_2] (Fig. 6.2 Process 3). This is supported by declines in total soil C and N content with [CO_2], indicating that microbial biomass either was similar between CO_2 levels or was reduced. Although this metric does not directly measure microbial biomass, it does indicate storage of C and N in soils declined and likely reflects a similar change in total microbial biomass C and N. Cumulative supply of mineral N over the experiment duration demonstrates that the association between mineral N availability and [CO_2] was non-linear

(Fig. 3.2). An increase of 75 µmol CO_2 mol^{-1} from 400 µmol CO_2 mol^{-1} resulted in a large jump in mineral N relative to the minor change in mineral N supply with a further increase of 75 µmol CO_2 mol^{-1}. What's more, the CO_2-effect on mineralisation of soil organic N indicates the input of mineral N was larger under 475 and 550 µmol CO_2 mol^{-1} than 400 µmol CO_2 mol^{-1}. Increased input of mineral N to soils under eCO_2 suggests the mechanisms driving declines in mineral between CO_2 levels may have been even stronger than is indicated by the differences between cumulative mineral N availability (Fig. 3.2).

Leaching may have impacted emission of N_2O by increasing loss of NO_3^- from soils (Fig. 6.2 Process 4). NO_3^- can be readily transported in saturated soils due to mobility of anions through negatively charged soil particles. Mineral N in TasFACE2 was predominantly NO_3^- and therefore was vulnerable to mobilisation through this process. Despite the sensitivity of leaching to soil water content (Di and Cameron 2002), there was no impact of irrigation, irrigation × [CO_2] interaction or WFPS on NO_3^- availability (Table 3.1). Furthermore, there is a horizon of medium to heavy clay at 30 cm at TasFACE2, which is an effective barrier, greatly impeding leaching of the soil solution. This may have led to the accumulation of NO_3^- at the loam-clay interface, a horizon well-within the rooting depth and susceptible to root uptake. Leaching may broadly be a concern in pastures as soil water content is increased under eCO_2 but was probably not a mechanism driving NO_3^- loss here.

NO and N_2O are produced as by-products during the oxidation of ammonium via nitrification (Fig. 6.2 Process 5). Nitrification is mediated by fungal nitrifiers and ammonia oxidising bacteria predominantly in aerobic soil conditions. Via laboratory incubation, potential net nitrification rates were determined to be extremely high but were unaffected by [CO_2] (Table 3.2). These rates were substantially increased in samples with urea addition compared to those without this addition. Therefore, nitrification rates were probably high under field conditions particularly following urea application. This indicates the likely possibility that

nitrification was a substantial contributor to NO and N_2O emissions here. However, there was a distinct decline in N_2O emissions under eCO_2 (Figs 4.1 and 4.2) which could have been a result of declines in nitrification rates or through complete reduction of NO and N_2O via denitrification. Nitrification requires oxygen and consequently the oxidation of NH_4^+ can be constrained by high water content. Although WFPS was increased under eCO_2, there was no effect of irrigation or CO_2 × irrigation effect on the availability of NH_4^+ or NO_3^-. Further the abundance of NO_3^- relative to NH_4^+ suggests nitrification rates were high regardless of WFPS. NO and N_2O produced via nitrification or denitrification can be completely reduced to N_2, an effect common when high water content limits gas diffusion through soil. Given analysis found no impact of WFPS on emissions, there is little evidence here that this occurred. Denitrification (Fig. 6.2 Process 6) was likely a major source of N_2O in this system and may help to explain the decline in emissions under eCO_2. Via denitrification, NO and N_2O are produced as intermediaries prior to the complete reduction of oxidised N to N_2. Denitrification mediated by heterotrophs occurs in anaerobic conditions and requires NO_3^- and organic matter. Plant responses to eCO_2 could affect this pathway in three ways: 1) greater plant water-use efficiency lowers soil aerobic status and promotes reduction 2) plant belowground C input increases heterotroph energy supply or 3) plant N immobilisation lowers mineral N available for reduction. First, the independent analysis of [CO_2], irrigation and the [CO_2] × irrigation indicated that the response of N_2O to [CO_2] was driven by plant N use and C deposition rather than changes in water use. Second, plant C deposition was undoubtedly increased under 550 µmol CO_2 mol^{-1} and possibly 475 µmol CO_2 mol^{-1} but the impact of this on denitrification is unclear given there was no stimulation of N_2O emissions with [CO_2]. Third, the NO_3^- supply was reduced substantially with [CO_2] due in part to greater plant N immobilisation (Fig. 3.1 and 3.2). The association between NO_3^- and N_2O emissions further demonstrates this as the primary mechanism by which [CO_2] altered N_2O

emissions. There is uncertainty as to what extent heterotrophic denitrification contributed to N_2O emissions in this system. The paradigm that denitrification is predominantly carried out in anaerobic environments by heterotrophs is being reconsidered given the past decades have revealed a wide range of fungi, bacteria and archaea capable of denitrification (Hayatsu et al. 2008). Some fungi and ammonia oxidising bacteria are capable of both nitrification and denitrification and can carry out these transformations over a wide range of soil water content (Zhou et al. 2001, Kool et al. 2011).These microbes have the potential to produce substantial quantities of NO, N_2O and N_2 in a variety of environmental conditions utilising NH_4^+ in nitrification and oxidised forms of N in denitrification. The reduction of NO_3^- and NO_2^- via denitrification is a common trait in fungi (Maeda et al. 2015), is insensitive to oxygen content relative to denitrification mediated by bacteria and can comprise up to 89% of total emissions in some grasslands (Crenshaw et al. 2008). The response of N_2O emissions to WFPS in TasFACE2 is uncharacteristic of denitrification mediated by heterotrophs and was probably driven primarily by nitrifiers. The determination of the N_2O sources in the grassland was beyond the scope of this study. However, future studies should consider investigation of the microbial community under eCO_2 as the findings from this study suggest nitrifier contributions to field emission of N_2O could be underestimated.

6.3 Concluding remarks

Rising atmospheric [CO_2] is one of the most pressing issues driving global change due to contributions to climate change but also due to the impact on biogeochemical cycles (IPCC 2014). Shifts in these cycles in future climate scenarios are expected to reduce the storage of C in the biosphere (van Groenigen et al. 2014) and accelerate the production of N_2O from soils (van Groenigen et al. 2011, Dijkstra et al. 2012). These forecasts pose challenges to management of the greenhouse gas budget due to the magnitude of natural fluxes relative to emissions associated with anthropogenic activities (Schlesinger and Andrews 2000, Thomson

et al. 2012). This thesis reveals that in this temperate pasture, micobially-mediated decomposition was stimulated and R_S was increased under eCO_2 but remained similar between eCO_2 levels. Given the relative increase in C stored in root biomass under 550 µmol CO_2 mol^{-1} was disproportionately greater than the increase in R_s under 550 µmol CO_2 mol^{-1}, it is possible that storage in root biomass may increase with [CO_2] though further study is required to determine how this affects long term C storage. Additionally, greater N immobilisation in plant biomass under eCO_2 due to root production resulted in lower NO_3^- availability and N_2O emissions in soils under eCO_2. Curiously, the CO_2-effect on N_2O emissions mediated by plants was non-linear over the three [CO_2], suggesting that increases in atmospheric [CO_2] did not have corresponding changes in N_2O emissions. The simultaneous control of [CO_2] and water supply allowed me to disentangle the direct effect of increased belowground plant C input and the indirect effect of increased water-use efficiency. In TasFACE2 there was a clear CO_2-effect on C and N cycles, but this effect was driven almost entirely by belowground C input rather than soil water supply. Additional consideration should be given to the design of FACE experiments in the future to test this finding more broadly, as most systems do not explicitly distinguish the role C cycling and water availability in eCO_2 experiments. TasFACE2 provided the opportunity to measure the CO_2-effect on C and N cycling at three rather than the usual two levels of most FACE studies. This allowed me to identify that the CO_2-effect on R_S and N_2O emissions may meet a threshold between 475 and 550 µmol CO_2 mol^{-1}, while the mineral N immobilised in plant biomass increased linearly between 400 and 550 µmol CO_2 mol^{-1}. Further study is required to determine if this is found broadly in other systems or if this is specific to this site. Collectively, the CO_2-effect on C and N cycling in this pasture demonstrates the importance of root responses to [CO_2] on soil processes. While this study is limited to observations in

1660 one pasture, it suggests the possibility that increased production of roots could modestly
1661 increase C storage in biomass and lower N_2O emissions in some systems.
1662

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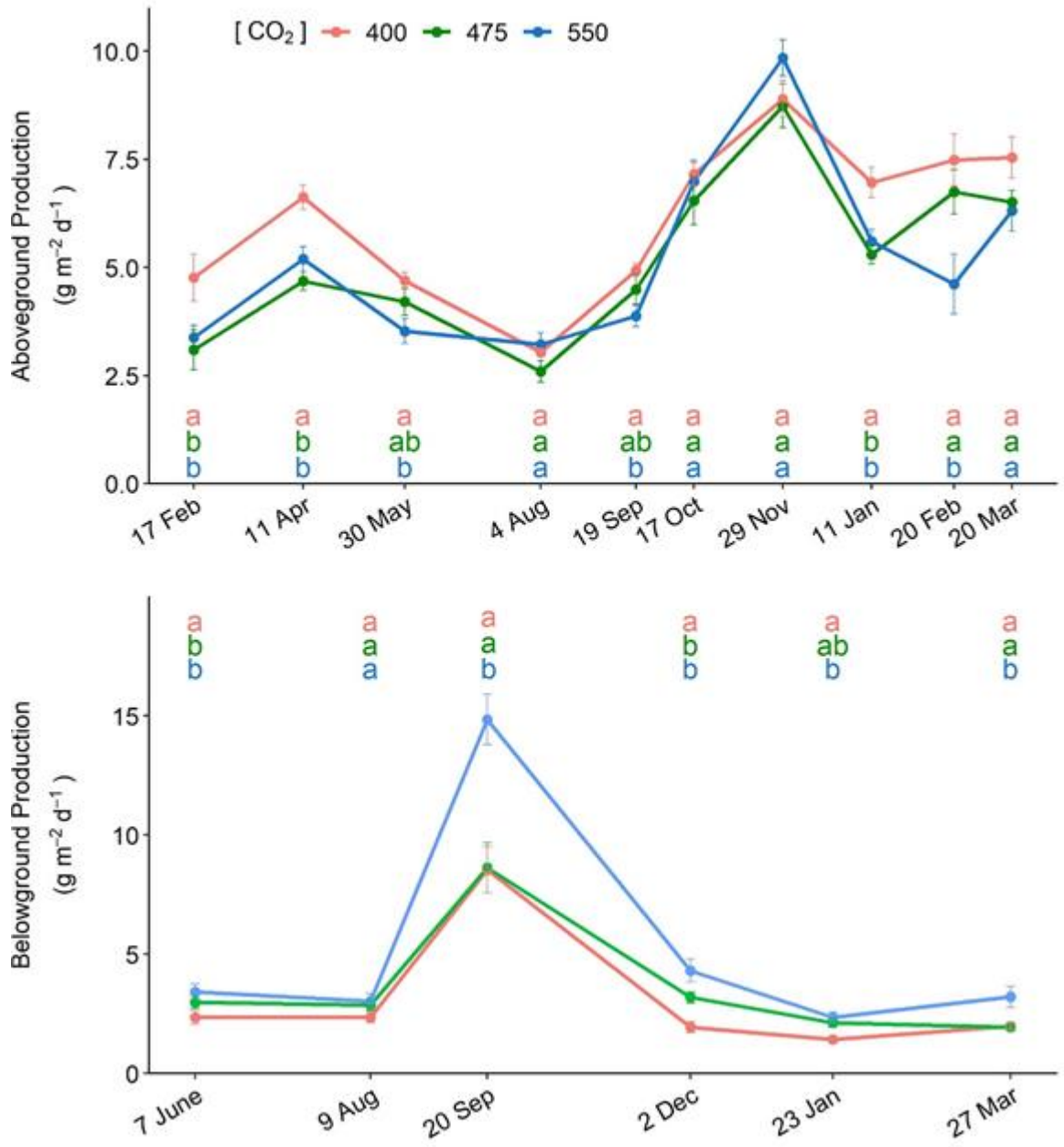
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. The effect of CO_2-treatment on daily production of aboveground (top) and belowground (bottom) biomass (g m^{-2} d^{-1} dry weight; mean ± SEM) in the TasFACE2 experiment between summer 2016 and summer 2017. Grass swards were exposed to three levels of CO_2 (400, 475, 550 µmol CO_2 mol^{-1}). Those means with the same letter within a date are not significantly different ($P > 0.05$).

Supplementary Table 1. Impact of [CO_2] (400, 475, 550 µmol CO_2 mol^{-1}) on soil respiration (R_S) from TasFACE2. Reported are estimated marginal means with SEM, degrees of freedom (df) and the lower and upper limits of the 95% confidence interval predicted using linear mixed effects models.

Month	[CO_2]	Mean	Effect size	SE	df	C.I. Lower	C.I. Upper
May	400	1.3	-	0.07	10	0.8	2.1
	475	1.8	0.4	0.07	10	1.1	2.7
	550	2.5	1.1	0.07	10	1.6	3.6
Sep.	400	3.4	-	0.07	10	2.3	4.7
	475	5.7	2.2	0.07	10	4.1	7.6
	550	6.3	2.7	0.07	10	4.6	8.4
Nov.	400	3.6	-	0.07	10	2.5	5.1
	475	7.7	3.8	0.07	10	5.8	10.1
	550	7.0	3.1	0.07	10	5.2	9.2
Jan.	400	3.3	-	0.07	10	2.2	4.6
	475	4.5	1.2	0.07	10	3.2	6.2
	550	4.9	1.5	0.07	10	3.5	6.6

Supplementary Table 2. Mean soil temperature º C ± SEM at 20 cm measured in plots under three levels of CO_2 (400, 475 and 550 µmol CO_2 mol^{-1}) and three irrigation volumes (*sufficient*, *limit*, and *excess*). Data averaged over 14-d soil respiration (R_S) campaigns in May 2016, September 2016, November 2016 and January 2017.

Soil Temperature (º C)							
Month	Irrigation	400 µmol CO_2 mol^{-1}		475 µmol CO_2 mol^{-1}		550 µmol CO_2 mol^{-1}	
May	*Limit*	11.7	±0.2	11.8	±0.2	11.8	±0.2
	Sufficient	11.7	±0.2	11.8	±0.2	11.7	±0.2
	Excess	11.7	±0.3	11.8	±0.3	11.8	±0.3
Sep.	*Limit*	13.0	±0.1	13.0	±0.1	13.0	±0.1
	Sufficient	13.0	±0.1	13.0	±0.1	13.0	±0.1
	Excess	13.0	±0.1	13.0	±0.1	13.0	±0.1
Nov.	*Limit*	16.0	±0.3	15.9	±0.3	15.9	±0.3
	Sufficient	16.0	±0.2	15.9	±0.2	15.9	±0.2
	Excess	16.0	±0.2	15.9	±0.2	15.9	±0.2
Jan.	*Limit*	19.6	±0.2	19.5	±0.2	19.5	±0.2
	Sufficient	19.5	±0.1	19.5	±0.1	19.5	±0.1
	Excess	19.5	±0.1	19.5	±0.1	19.5	±0.1